WOLBACHIA SYMBIOSIS IN THE AGRICULTURALLY IMPORTANT PREDATORY MITE *METASEIULUS OCCIDENTALIS*

By

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Abstract of Dissertation Presented to the Graduate School
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This study focused on detecting, describing, and evaluating the biological effects of *Wolbachia* endosymbionts in the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Acari: Phytoseiidae). *Wolbachia* were found in adults and eggs of *M. occidentalis* using *Wolbachia* specific polymerase chain reaction (PCR) primers which amplify the 16S ribosomal RNA and *ftsZ* genes. *Wolbachia* also were found in their prey, the two spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae). *Wolbachia* DNA from the two mite species was sequenced and compared. Parsimony analysis indicated the mite *Wolbachia* sequences were very similar to one another and to *Wolbachia* from insects. *Wolbachia* DNA could be transiently detected in the uninfected predatory mite *Amblyseius reductus* Wainstein (Acari: Phytoseiidae) after feeding on infected spider mites, indicating the infection status of any predatory arthropod's diet should be considered before using the PCR to detect *Wolbachia*.

In insects, *Wolbachia* causes nonreciprocal reproductive incompatibilities in crosses between infected males and uninfected females the reciprocal crosses are normal Genetically similar populations of *M occidentalis* differing in the presence of *Wolbachia* (due to heat-curing of one population) were crossed to assess the effects of the symbiont *Wolbachia* did induce non reciprocal incompatibilities in this parahaploid mite, evidenced by significantly fewer viable eggs higher proportions of egg shriveling as compared to the reciprocal and control crosses, and no or few female progeny

To determine whether the proportion of *Wolbachia*-infected *M occidentalis* in a polymorphic population would increase over time (because infected females can reproduce with both infected and uninfected males), three *M occidentalis* populations were initiated with 10% infected and 90% cured mites and monitored for 12 generations *Wolbachia* infection did not spread rapidly through the populations Imperfect transmission rates and fitness costs were detected, which may prevent the rapid spread of *Wolbachia* This suggests *Wolbachia* would not be useful as a "drive mechanism" for inserting useful genes into field populations of *M occidentalis*

CHAPTER 1
INTRODUCTION

Historical Sketch

Wolbachia bacteria were first described from the gonadal tissues of the mosquito *Culex pipiens* L. in 1924 by Hertig and Wolbach, the type species is therefore named *Wolbachia pipientis* Hertig. Strange reproductive incompatibilities were later described in *Culex pipiens* mosquitoes by Ghelelovitch (1952) and Laven (1951). One type of incompatibility was nonreciprocal, meaning that crosses of males from population A with females of population B resulted in normal progeny, but crosses of males from population B with females from population A (the reciprocal cross) resulted in few viable progeny. The phenomenon was named "cytoplasmic incompatibility" (Laven 1959). In the 1970s Yen and Barr (1971) first correlated these nonreciprocal cytoplasmic incompatibilities with the presence of *Wolbachia* endosymbionts. They found that when *Wolbachia*-infected males were treated with tetracycline (which is toxic to rickettsia like microorganisms), they could reproduce successfully with uninfected females. Because *Wolbachia's* morphological characters are of limited value and *Wolbachia* are difficult to culture outside the host (Weiss and Moulder 1984, O'Neill *et al.* 1992), their presence in other arthropods was merely speculative.

In 1992, *Wolbachia*-specific polymerase chain reaction (PCR) primers were developed (O Neill *et al* , 1992) These primers were designed to be specific to *Wolbachia*, while at the same time general enough to amplify *Wolbachia* 16S ribosomal DNA from various insects Reproductive anomalies associated with the presence of an unknown rickettsia could now be correlated with the presence of *Wolbachia* For example *Wolbachia* infection was confirmed in some California populations of *Drosophila simulans* Sturtevant (O Neill *et al* , 1992) This symbiont was previously suspected to be the causative agent of nonreciprocal reproductive incompatibilities between geographical populations of this insect (Hoffmann *et al*., 1986) Because uninfected females are reproductively incompatible with infected males, and infected females can reproduce successfully with infected and uninfected males infected females tend to have a reproductive advantage in polymorphic populations (Caspari and Watson, 1959, Turelli and Hoffmann, 1991) In fact the *D simulans Wolbachia* infection has spread within and among California populations (Turelli and Hoffmann, 1991 Turelli *et al* 1992) since it was first documented in 1986 (Hoffmann *et al* 1986) The increased proportion of infected individuals is presumably due to the reproductive advantage afforded to infected females (Turelli and Hoffmann 1991) Several parameters determine the ability of *Wolbachia* to spread through a population, including the stability of infection as a function of maternal transmission frequency fitness costs associated with infection and the strength of incompatibility (Hoffmann *et al* , 1990 Turelli *et al* 1992, Clancy and Hoffmann 1997)

Efforts are under way to genetically engineer insects' mutualistic endosymbionts to be refractory to disease agents like those causing malaria or Chagas' disease (Beard *et al.* 1993). These transformed arthropods need a way to replace the wild-type insects already present in the field population. The ability of *Wolbachia* to spread through a population, as documented in *D. simulans*, could be harnessed as mechanism to help drive a genetically altered symbiont through a population if it "hitchhikes" with the *Wolbachia* infected cytoplasm (Caspari and Watson, 1959; Beard *et al.* 1993).

Wolbachia Biology

A fuller understanding of *Wolbachia* biology is necessary before it can be used successfully as a drive mechanism (Werren, 1997). What is known about these endosymbionts is that they are intracellular, rickettsial-like endosymbionts in the alpha-subdivision of the proteobacteria (purple bacteria) (for a review of the biology of this symbiont and the diversity of hosts it infects, see Werren, 1997). *Wolbachia* are transmitted through the egg cytoplasm, therefore transmitted solely by females. There was, however, one reported case of male transmission in laboratory populations of *D. simulans* (Hoffmann and Turelli, 1988). *Wolbachia* are sensitive to high temperatures (Stevens, 1989; Stouthamer *et al.*, 1990; Ginn and Bouletreau, 1995; Louis *et al.* 1993), and the antibiotics rifampin and tetracycline (Stouthamer *et al.* 1990a). The only success to date in culturing them outside the host has been in an *Aedes albopictus* (Skuse) cell line (O'Neill *et al.* 1995).

Because they cannot be studied using traditional microbiological techniques (Weiss and Moulder 1984), the PCR and DNA sequencing has provided a major breakthrough in their study (O'Neill *et al*., 1992 Breeuwer *et al*., 1992, Rousset *et al*., 1992a Stouthamer *et al*., 1993) The PCR allows the amplification of a specific region of *Wolbachia* DNA more than a million-fold The presence or absence of the symbiont then can be determined by visual detection of the expected size fragment of DNA in an ethidium bromide-stained agarose gel under UV light This amplification also yields ample DNA for sequencing and further description and characterization DNA sequence analyses indicate a lack of concordance between the phylogenies of the symbiont and of the hosts suggesting this symbiont might sometimes be transmitted horizontally from species to species (Rousset *et al*., 1992a, O'Neill *et al*., 1992) Recent studies using the PCR determined that 16% of all insect species examined are infected with *Wolbachia* (Werren *et al*., 1995a)

The effects of *Wolbachia*, for example the nonreciprocal reproductive incompatibilities detected in *C. pipiens* and *D. simulans* are influenced by several factors The strain of *Wolbachia* is important some strains have been demonstrated to cause no reproductive alterations (Giordano *et al*., 1995) The phenotype of *Wolbachia*-mediated reproductive alterations also depends on the taxonomic status of the affected arthropod (Insecta, Arachnida, Isopoda) (see Werren 1997), as well as the genetic system of the arthropod It is important to consider arthropod genetic systems in order to better appreciate the diversity of *Wolbachia's* effects on reproduction

Diplo-diploid arthropods produce both sexes from fertilized eggs each sex carrying both the maternal and paternal sets of chromosomes

throughout their lives. In haplo-diploid arthropods, female progeny arise from fertilized eggs and are diploid, but the male progeny arise from unfertilized eggs and are haploid, carrying only the maternal set of chromosomes. Thelytoky is a genetic system in which virgin females produce diploid daughters parthenogenetically, rarely producing males. In a parahaploid genetic system, both sexes initially arise from fertilized (diploid) eggs with both sets of chromosomes. However, one chromosome set is subsequently lost in males, and the adult male is haploid, producing sperm by a mitotic process.

When males of infected diplo-diploids mate with females lacking *Wolbachia*, the paternal chromosome set becomes abnormal in the fertilized egg (Kose and Karr, 1995; O'Neill and Karr, 1990), resulting in the death of both male and female progeny (Hoffmann *et al.*, 1986; Hsiao and Hsiao, 1985). The reciprocal cross is normal. Although the molecular mechanism of this incompatibility is not yet fully understood, it is speculated that *Wolbachia* somehow "imprints" or "modifies" the paternal set of chromosomes (Werren, 1997). If *Wolbachia* is present in the egg cytoplasm, it can "rescue" the paternal chromosomes so that they remain normal and produce the normal diploid sons and daughters. If no *Wolbachia* is present, there is no "rescue" and those paternal chromosome set becomes abnormal, leading to embryonic death.

This same mechanism may occur in haplo-diploid insects, but with different consequences. When infected haploid males mate with uninfected diploid females, the male (haploid) progeny remain normal, but the normally diploid female embryos become haploid due to abnormalities in the paternal set of chromosomes (Ryan and Saul, 1968; Reed and Werren,

1995) The resulting phenotype of *Wolbachia*-mediated incompatibilities in haplo-diploid species is a strongly male-biased sex ratio because of the loss of female progeny The haploid female embryos may die as in some strains of the two-spotted spider mite *Tetranychus urticae* Koch (Chelicerata Arachnida) (Vala and Breeuwer, 1996) or the haploid female embryos can become males thereby increasing the total number of expected males, as in the jewel wasp *Nasonia vitripennis* Walker (Mandibulata Insecta) (Breeuwer and Werren 1990 Ryan and Saul 1968)

Wolbachia also can cause bidirectional incompatibility in diplo-diploid species (O Neill and Karr 1990) and haplo-diploid species (Ferrot Munot *et al* , 1996) In this situation, two populations apparently host two different *Wolbachia* strains The result is reciprocal incompatibility where both interpopulation crosses are incompatible

Wolbachia induces thelytoky in some hymenopteran species such as *Trichogramma* (Stouthamer *et al* 1990b) and *Aphytis* (Zchori-Fein *et al* , 1995) *Wolbachia* allows these females to produce diploid daughters parthenogenetically by causing what is termed by Stouthamer and Kazmer (1994) as "gamete duplication" early in the first mitotic division. This phenomenon is probably described more accurately as duplication of the chromosomes in the oocyte

Wolbachia causes a typical diplo-diploid incompatibility phenotype in some isopods (Rousset *et al* , 1992b), as well as an unusual phenotype in the species *Armadillium vulgare* Latr In this species, *Wolbachia* suppresses the androgenic gland in genetically male individuals causing these male isopods to become functional females (Rigaud et al 1991) It is likely that, with the

diversity of *Wolbachia's* effects on the arthropod taxa and genetic systems described to date, there may be more *Wolbachia* mediated reproductive anomalies remaining to be described

Wolbachia may have other important ecological and evolutionary effects *Wolbachia*-mediated reproductive isolation may be one mechanism that could allow sympatric speciation to occur (Laven 1959, Werren, 1997) *Wolbachia* alters sex ratios and progeny survival and, as a consequence may affect laboratory experiments and insect management in field programs For example, incompatibilities may interfere with crosses conducted during hybridization studies..one method of determining species designations in some insects and phytoseid mites (Croft 1970 McMurtry *et al*., 1976, McMurtry 1980 McMurtry and Badii, 1989) Studies on the mode of inheritance of pesticide resistance have been affected by cross incompatibilities (Hoy and Knop, 1981, Hoy and Standow, 1982) *Wolbachia* infection may have implications for mass rearing projects especially if the bacteria have an influence on the quality of the natural enemies (Steiner, 1993) or affect the rate of population increase of the individuals being reared

Wolbachia in the Predatory Mite, *Metaseiulus occidentalis*

The western predatory mite *Metaseiulus* (=*Typhlodromus* or *Galendromus*) *occidentalis* (Nesbitt) is a useful natural enemy of *Tetranychus* species including the two spotted spider mite *Tetranychus urticae* This predatory mite is used as a biological control agent in various crops in the western United States (Hoyt, 1969, Hoyt and Caltagirone 1971), including

apples (Hoyt 1969- Hoyt and Caltagirone, 1971) peaches (Hoyt and Caltagirone, 1971) grapes (Flaherty and Huffaker 1970) and almonds (Hoy 1985) Figure 1-1 shows an adult *M occidentalis* feeding on an adult *T urticae* For lists of references on *M occidentalis* and other phytosends, see Hoy (1982) and Kostiainen and Hoy (1996)

The effectiveness of *M occidentalis* can be negatively affected by chemical sprays used to control insect pests occurring within the same agricultural ecosystem Genetically improved strains of this predator that are resistant to various chemical pesticides have been developed (reviewed by Hoy 1985) and utilized as part of integrated pest management programs to control spider mites (Hoy *et al* , 1982) already resistant to many of these chemicals In addition, efforts are under way to develop recombinant DNA techniques to further improve these and other natural enemies for use in agriculture (Presnail and Hoy 1992).

Details about the biology behavior ecology, and genetics of *M occidentalis* have been and remain important to better understand and improve this predator as a biological control agent in agriculture (Hoy, 1985) Of particular interest are biological characteristics which affect their rate of population increase in the field and in the rearing laboratory (Sabelis, 1985) Studies on the genetic system, mating behavior, sex ratio, and reproductive incompatibilities of *M occidentalis* have yielded a great deal of information on the reproductive biology of these predators, while at the same time have raised several interesting questions

Studies on the reproductive biology of the phytosends *Phytoseiulus persimilis* Athias-Henriot (Helle *et al* , 1978), *Amblyseius limens* Blommers

(Helle *et al* 1978), and *M occidentalis* (Hoy 1979) revealed their unique genetic system called parahaploidy (also referred to as "pseudo-arrhenotoky" by Schulten (1985) to distinguish it from the similar system found in insects) Hoy (1979) found that in studies in which X irradiated males were mated with unirradiated females, only sons were produced Any sons produced were low in number and sterile suggesting these males are derived from fertilized eggs, beginning life as a diploid, and later losing half of their chromosome set sometime during embryonic development (Hoy 1979) Nelson-Rees *et al* (1980) demonstrated cytologically that both male and female *M occidentalis* are diploid at the beginning of embryonic development, but at the onset of the reductional division 24-48 hours after egg deposition, one of the sets becomes heterochromatinized and excluded from the nucleus. Studies on the inheritance of pesticide resistance in *P persimilis* by Helle *et al* (1978) and in *M occidentalis* by Hoy and Standow (1982) and Roush and Plapp (1982) suggest the paternal set of chromosomes is lost although recent use of RAPD-PCR DNA markers suggests that some of the paternal genome may be retained in males of the phytoseiid *Typhlodromus pyri* Scheuten (Ferrot-Minnot and Navajas, 1995) More data are needed to verify whether this is true and to further clarify the mechanism of parahaploidy in these and other phytoseiids

Sex allocation and the resultant sex ratios of phytoseiids have practical consequences for biological control and associated mass rearing projects (Sabelis 1985) Amano and Chant (1978) suggest there is a characteristic sex ratio between 50 and 100 percent females for each phytoseiid species These sex ratios differ between the species but are fairly consistent within species

(Sabelis 1981) This consistency has been contradicted by others who have reported intraspecific variation in several species Croft (1970) detected sex ratio differences in the colonies and differences in crosses between colonies of *M occidentalis* from Utah California and Washington. Roush and Hoy (1981) found that a carbaryl resistant strain of *M occidentalis* crossed with a susceptible colony produced progeny with sex ratios different from those of each strain mated *inter se* The resistant strain had a higher sex ratio (more females) than the susceptible McMurtry *et al* (1976) found that the sex ratio varied in reciprocal crosses of geographical races of *Amblyseius potentillae* Garman Variation in sex ratios of different geographical populations and colonies suggests a genetic component to sex ratio (Hoy, 1982) There have been correlations also between temperature and sex ratio (Dyer and Swift, 1979 Tanigoshi *et al* 1975), relative humidity and sex ratio (Dyer and Swift 1979) starvation and sex ratio (Tanigoshi *et al* 1975), and mating duration and sex ratio (Schulten *et al* , 1978 Elbadry and Elbenhawy 1968, Amano and Chant, 1978)

There also have been reports of partial reproductive incompatibilities between strains of the same phytoseiid species Associated with these reproductive incompatibilities were shriveled eggs low numbers of eggs, low survival of immature stages, and reduced fecundity in surviving F_1 individuals (Croft, 1970 Hoy and Knop 1981 Hoy and Standow 1982, Hoy and Cave, 1988) Croft (1970) detected reciprocal reproductive incompatibilities (females from both of the two populations being crossed are incompatible with males from the different population) and nonreciprocal reproductive incompatibilities (females from only one of the two populations

being crossed are incompatible with males from the other population) in crosses of *M. accidentalis* from California, Utah, and Washington. When Hoy and Knop (1981) crossed a laboratory selected permethrin resistant strain of *M. accidentalis* with its original base colony, few eggs were produced and many were shriveled and failed to develop in one cross while the reciprocal cross was compatible. They also found that a partially permethrin resistant strain was nonreciprocally incompatible with its base colony after only one year of laboratory selections. Similar nonreciprocal reproductive incompatibilities were found in crosses with sulfur resistant *M. accidentalis* (Hoy and Standow 1982). In a later study, Hoy and Cave (1988) detected nonreciprocal partial reproductive incompatibilities between five other colonies of *M. accidentalis*. Other examples of nonreciprocal incompatibilities in phytoseiids were found in *Typhlodromus annectens* DeLeon (McMurtry and Badii, 1989) and in two populations of *Amblyseius addoensis* van der Merwe and Ryke from South Africa (McMurtry 1980).

The cause of the nonreciprocal reproductive incompatibilities was unknown in these phytoseiids. They were somewhat similar to the nonreciprocal incompatibilities previously described in the mosquito *C. pipiens* L. (Ghelelovitch 1952, Laven, 1951) determined to have a cytoplasmic inheritance pattern (Laven 1959). A cytoplasmic inheritance pattern is suspected when the nuclear genetic makeup of the hybrids is virtually the same but the main difference is which mother's cytoplasm is present. An intracellular rickettsia-like microorganism was found by Hess and Hoy (1982) in *M. accidentalis* eggs and ovaries through light and electron microscopy. This observation, along with the nonreciprocal nature of the incompatibilities

led Hoy and Cave (1988) to speculate that a cytoplasmic factor may be responsible for the observed reproductive aberrations seen in *M. occidentalis* perhaps due to the presence of *Wolbachia*.

I decided to investigate *Wolbachia* in *M. occidentalis* because of this predator's importance as a biological control agent because it has a genetic system that has not yet been studied in relation to *Wolbachia*-mediated incompatibilities, and because of the potential to use *Wolbachia* as a drive mechanism in genetic improvement programs. In addition, this mite has been used as a model organism in past experimental ecological simulations due to its rapid generation time and ease of rearing (Huffaker, 1958).

Research Goals

The first goal was to determine whether *Wolbachia* was present in *M. occidentalis* (Chapter 2). The PCR was used to detect this endosymbiont in various laboratory and field populations of *M. occidentalis* and its prey *T. urticae*. The amplified 16S rDNA was sequenced and comparisons were made between the *Wolbachia* sequences found in both predator and prey mites and the *Wolbachia* sequences from various insects. The final goal of Chapter 2 was to determine whether starvation could eliminate false positive PCR signals in predators fed on *Wolbachia* infected prey.

Wolbachia 16S ribosomal DNA sequences from *M. occidentalis* and *T. urticae* were highly similar (Chapter 2). PCR primers have recently become available that amplify other *Wolbachia* genes, such as the *ftsZ* gene and the

surface protein gene. Attempts were made to further characterize *Wolbachia* in *M. occidentalis* and *T. urticae* by using these genes (Chapter 3).

Once *Wolbachia* were identified, correlations between the presence of *Wolbachia* and their influence on reproduction in *M. occidentalis* were investigated (Chapter 4). Genetically similar inbred lines differing only in the presence or absence of *Wolbachia* were crossed and evaluated to determine whether this symbiont is associated with nonreciprocal reproductive incompatibilities in crosses between infected males and heat cured females. The incompatibility phenotype would be a useful model of *Wolbachia* induced reproductive alterations in a parahaploid arthropod.

To determine whether *Wolbachia* could spread through a polymorphic population due to the reproductive advantage afforded to infected females, laboratory populations initiated with 10% infected and 90% uninfected eggs were monitored for 12 generations. The stability of infection was also assessed by analysis of the control populations (Chapter 5).

Chapter 6 provides a general discussion of the results of my research. I will address what I have learned about *Wolbachia*, some implications of the results, and potential directions of future research.

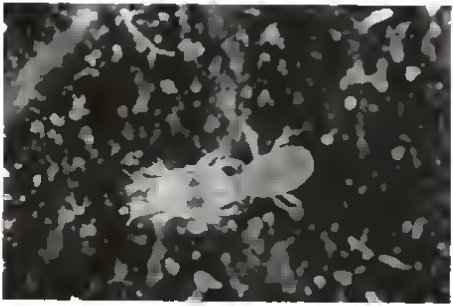


Figure 1.1 An adult female western orchard predatory mite, *Metaseiulus occidentalis*, is pictured on the right feeding on an adult two-spotted spider mite *Tetranychus urticae*

CHAPTER 2
16S RIBOSOMAL DNA ANALYSIS OF *WOLBACHIA* FROM TWO PHYTOSEIIDS (ACARI: PHYTOSEIIDAE) AND THEIR PREY (ACARI: TETRANYCHIDAE)

Introduction

Wolbachia are rickettsia like microorganisms in the alpha subdivision of the proteobacteria which are intracellular maternally inherited, and found in the gonadal tissues of some insects. They were detected originally in the northern house mosquito, *Culex pipiens* L. (Hertig, 1936, Yen and Barr, 1971), the type species is named *Wolbachia pipientis* Hertig. *Wolbachia* has been reported to have negative (Stevens and Wade, 1990, Horjus and Stouthamer 1995) zero (Moran and Bauman 1994, Poinsot and Merçot, 1997), or positive (Wade and Chang, 1995) effects on host fitness so it remains unresolved whether *Wolbachia* symbionts are mutualists or parasites.

Wolbachia are associated with unidirectional and bidirectional intraspecific mating incompatibilities in many insects (Yen and Barr 1973, O'Neill and Karr, 1990, Breeuwer and Werren, 1990) and thelytoky in some parthenogenetic insects (Stouthamer *et al* 1993). Infected females may have a reproductive advantage when other females in the population are not infected. Females with *Wolbachia* can produce viable progeny when mating with both infected and uninfected males. Uninfected females are partially incompatible with infected males and therefore produce fewer progeny than females infected with the same strain of *Wolbachia* which can lead to the

elimination of uninfected individuals in the population over time Bidirectional mating incompatibilities caused by *Wolbachia* infections may play a role in the sympatric speciation of arthropods through reproductive isolation (Laven, 1959)

The polymerase chain reaction (PCR) can be used to detect *Wolbachia* in insects when specific oligonucleotide sequences (primers) complementary to a portion of *Wolbachia* 16S ribosomal DNA (rDNA) are used (O'Neill *et al* 1992) Visual detection of these amplified fragments can be made after gel electrophoresis and staining with ethidium bromide The PCR products can be sequenced to confirm the identity and phylogenetic placement of the DNA Often 16S ribosomal DNA sequences are used to reconstruct bacterial phylogenies because they are highly conserved in prokaryotes (Woese, 1987) *Wolbachia* 16S rDNA sequences from various insect orders are available through the GenBank and European Molecular Biology Laboratories (EMBL) databases including those from the Diptera (O'Neill *et al* , 1992 Rousset *et al* 1992a) Hymenoptera (Breenwer *et al* 1992 Stouthamer *et al* , 1993) Coleoptera, and Lepidoptera (O'Neill *et al* 1992)

The predatory mite *Metaseiulus* (=*Typhlodromus* or *Galendromus*) *occidentalis* (Nesbitt) (Acari: Phytoseudae) is a natural enemy of spidec mites, including the two spotted spider mite *Tetranychus urticae* Koch *M occidentalis* is used as a biological control agent in a variety of crops in the western United States (Hoyt, 1969 Hoyt and Caltagirone, 1971) Previous studies have been conducted on the cytogenetics of *M occidentalis* (Nelson Rees *et al* 1980) and on rickettsia like microorganisms (Hess and Hoy, 1982), which were found in eggs and ovaries of *M occidentalis* Furthermore

observations of nonreciprocal partial reproductive incompatibilities between certain populations (Croft, 1970; Hoy and Knop 1981 Hoy and Standow, 1982, Hoy and Cave, 1988) suggested the possibility of infection by *Wolbachia* Previous studies (e g, Overmeer and van Zon 1976, deBoer 1982) have also demonstrated that cytoplasmic incompatibilities occur in the two spotted spider mite, suggestive of *Wolbachia* infection

One difficulty in detecting and subsequently sequencing *Wolbachia* DNA in *M accidentalis* is that their prey could also contain *Wolbachia* The pollen feeding phytoseiid *Amblyseius reductus* Wainstein is not infected with *Wolbachia* (unpublished data) and therefore can be used to test whether false positive PCR signals caused by transient gut contents could be eliminated methodologically

The goals of this study were to 1) conduct a survey by using the PCR to detect the presence of *Wolbachia* in several predator and prey populations, 2) determine whether the otherwise *Wolbachia* free predator *A reductus* becomes positive for *Wolbachia* as determined by the PCR after feeding on prey containing *Wolbachia*, 3) evaluate starvation as a method for eliminating false positive PCR signals in *Wolbachia*-free predators fed prey containing *Wolbachia* and 4) to use parsimony analysis to compare the 16S rDNA sequences obtained to the *Wolbachia* sequences from various insects to confirm the identity of the DNA as *Wolbachia* 16S rDNA.

Materials and Methods

Colony Sources and Maintenance

A series of seven laboratory colonies (Table 2-1) of *Metaseiulus occidentalis* (COS Russian Select, Supermite Hybrid Select, Ave-21 Pullman Blackberry, WA Select) maintained at the University of Florida and reared as previously described (Roush and Hoy, 1981 Hoy *et al*., 1982) were surveyed for the presence of *Wolbachia* by the PCR. One *M. occidentalis* laboratory colony from Oregon and one insectary colony from California were sampled immediately after they were received. Field-collected *M. occidentalis* from Washington apples and their prey, European red mite *Panonychus ulmi* (Koch), were collected and subsequently reared on detached apple leaves in our laboratory for use in the PCR survey. The *A. reductus* used in the time course study were reared in the laboratory on a diet of cattail *Typha latifolia* L. pollen, but will eat the two spotted spider mite *Tetranychus urticae* if provided. A colony of two spotted spider mites was raised on pinto bean *Phaseolus vulgaris* L. plants in a greenhouse at the University of Florida-Gainesville and later used in the PCR survey. *T. urticae* were also obtained from a laboratory colony in Oregon, a laboratory colony from Ohio, and an insectary in California. A population of *T. urticae* and of the strawberry spider mite, *T. turkestani* Ugarov and Nikolski were collected from a field of cotton in California. The *T. urticae* and *T. turkestani* obtained from other sources were maintained on detached bean leaves in the laboratory.

DNA Extraction

All predators were starved between 4 and 8 h prior to DNA extractions, and mites from the newly acquired predatory mite colonies were tested before feeding on *T. urticae* from our laboratory. DNA from the Russian Select adult female *M. occidentalis* and the male *T. urticae* used for the PCR and subsequent direct sequencing was extracted by a modified version of the technique reported by Edwards and Hoy (1993). Fresh, not frozen, individual and pooled adults (5 mites) were macerated in 50 µl of a 5% Chelex (Bio-Rad, Hercules, CA) solution, heated to 56°C for 30 min (instead of 15 min), then to 95°C (instead of 100°C) for 8 min. The samples were centrifuged briefly and stored at -20° C before the PCR.

Some inconsistencies in results were noticed by using the Chelex method, so the DNA from the eggs of COS *M. occidentalis* used for sequencing, as well as the DNA used for all other PCR surveys and experiments, was extracted as follows. DNA from eggs and adults was extracted by macerating 5 adult mites or 60 eggs in 25 µl of STE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA pH = 8.0) and 1 ml of proteinase K (10 mg/ml) (O'Neill *et al.* 1992). Samples were macerated with a new glass pestle for each sample in a 1.5 ml Eppendorf tube. Pestles were made by flaming the tip of a pasteur pipette and bending it slightly to form a rounded end. The preparation was heated to 95°C for 8 min, briefly centrifuged, and used immediately for the PCR reactions.

Polymerase Chain Reaction Conditions

All reactions were run with the following conditions: 50 mM KCl, 10 mM Tris HCl, 2.5 mM $MgCl_2$, 0.2 µM each primer, 200 µM each dNTP, and 0.8 units of *Taq* in a total volume of 25 µl. The initial 16S rDNA primers were provided by S. O'Neill (O'Neill *et al.*, 1992), and subsequent primers were synthesized by the University of Florida DNA Synthesis Laboratory. The primers correspond to *E. coli* positions 76-99 forward (5'-TTGTAGCCTGCTATGGTATAACT) and 1012-994 reverse (5'-GAATAGGTATGATTTTCATGT), and produce a PCR product of ~900 bp. Reactions were cycled 40 times at 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s, followed by a 4 min extension period. The amount of template used varied according to species and life stage, but was usually 5 µl. Reagent negative controls were included in the reactions. Positive controls were *Drosophila simulans* Sturtevant DNA (supplied by R. Giordano, University of Illinois Urbana) or dilutions of previously amplified DNA from *M. occidentalis* with *Wolbachia* specific primers. The camel's-hair brush used to transfer the mites to the pinto bean leaves and roots, and the debris (exuviae, dead spider mites) in the predator colonies were tested by PCR for contaminating *Wolbachia* DNA. PCR products were electrophoresed in a 1.5% agarose gel in TBE/EtBr for 90 min at 60 mV, then photographed on a UV transilluminator.

Effects of Feeding Predators With *Wolbachia*-Positive Prey

A study was done to determine whether the otherwise *Wolbachia*-free *A. reductus* would become positive for *Wolbachia* by the PCR after feeding on *T. urticae* containing *Wolbachia*, or whether the positive PCR signal seen from

adult female *M. occidentalis* would disappear when removed from a *T. urticae* food source. Adult females of both species were fed a diet of *T. urticae* for at least 5 d and later placed in an arena without prey. The predators were then killed 0, 4, 8, 16, 24, and 48 h later by placing them into a -80°C freezer, where they were stored until their DNA was extracted for PCR analysis. If whole mites were frozen, preliminary tests indicated PCR amplification could be conducted with consistent results. The DNA from 5 mites was combined per replicate, and 5 replicates were done for each time point, with the exception of 3 replicates for *A. reductus* at 48 h after feeding. The DNA extractions and PCR reactions from 3 of the 5 replicates from all time periods were done on one day, the remaining 2 samples from each time period were analyzed on the following day. Negative controls were water and DNA from *A. reductus* never fed *T. urticae*. *Drosophila simulans* DNA was a positive control.

DNA Purification and Sequencing

The PCR product from one *M. occidentalis* female (Russian Select strain) starved for 8 h was reamplified and purified with a QIAquick Spin PCR Purification Kit (QIAGEN, Chatsworth, CA) for direct sequencing. Similarly, the PCR product from one male *T. urticae* was reamplified and purified for subsequent direct sequencing. The PCR product from 60 *M. occidentalis* eggs (COS strain) was also reamplified and purified. The reamplified egg DNA was purified by extracting the 900-bp band from an agarose gel by using a QIAquick Gel Extraction kit (QIAGEN). The PCR products were sequenced by the ICBR DNA Core Sequencing Facility at the University of Florida with an ABI 373a Automated Sequencer.

Phylogenetic Analysis

The 16S rDNA sequences from microorganisms including *Wolbachia* were aligned with the mite sequences by eye by using conserved areas as markers. Parsimony analyses with PHYLIP (Felsenstein, 1993) and MacClade (Madisson and Madisson, 1992) were used to compare our sequences with the other aligned sequences. *Bacillus subtilis* Cohn was used as an outgroup. Many reported *Wolbachia* sequences are shorter than those I sequenced; therefore a 625-bp sequence from the 5' end of the 16S rDNA gene (*E. coli* positions 100-773) was used in the phylogenetic analyses.

Heat Treatment

Temperatures >30°C administered for a few generations have been reported to eliminate *Wolbachia* from some insects (Stevens, 1989; Stouthamer *et al.*, 1990a). One colony of *M. accidentalis* (COS) was reared at 33°C for at least 6 generations to determine whether it is possible to decrease their *Wolbachia* to undetectable levels.

Results

DNA Extraction

Although DNA preparations with Chelex yielded some positive PCR signals, the STE preparation method (O'Neill *et al.*, 1992) gave more consistent results, especially when the DNA preparation was used immediately after the extraction and was never frozen.

Polymerase Chain Reaction Assay

The survey of predator and prey populations indicates the 900-bp *Wolbachia*-specific PCR product can be amplified from laboratory colonies of *M. occidentalis* and *T. urticae* but not amplified from the field populations tested (Table 2-1). However, a field population of the other spider mite tested, *T. turkestani*, which was collected from the same cotton field containing an uninfected population of *T. urticae*, was positive. The European red mite, prey of the freshly collected field population of *M. occidentalis* tested, was also negative by the PCR. The laboratory colony of *A. reductus* remained uninfected during the course of the study. Negative controls, the camel's-hair brush, pinto bean material, and the debris were negative throughout the study. Some nonspecific PCR products were present in some reactions and excluded from analyses.

The PCR was performed on egg preparations of selected *M. occidentalis* strains to eliminate the possibility of false positive signals caused by adults feeding on positive *T. urticae* prey. When the adults were positive, the eggs were also positive (Table 2-1) (COS, Russian Select, Pullman Blackberry). Likewise, when the adults were negative, the eggs were negative (Supermite, Griggs Apple). Figure 2-1 (lanes 2–4) shows the PCR results from COS adults and eggs. The presence of *Wolbachia* from egg preparations suggests that *Wolbachia* are transovarially transmitted, not just present as gut contaminants from feeding on positive *T. urticae*. Transovarial transmission of the type B rickettsia-like microorganism in *M. occidentalis* was predicted previously because of their primary presence in the ovaries of adult females.

(Hess and Hoy, 1982) and because of the presence of small microorganisms observed in egg squashes of *M. occidentalis* (Nelson Rees *et al*., 1980)

Effects of Feeding Predators Infected Prey

A. reductus adult females fed *T. urticae* were positive for *Wolbachia* by the PCR in only one replicate of five when tested immediately after the predators were removed from positive prey. All the remaining samples were negative. Positive and negative controls indicated that the PCR reactions were reliable. In *M. occidentalis* positive PCR signals were detected in all five samples tested 0 and 4 h after feeding, in four of the five samples 8, 12, and 24 h after feeding, and in three of the five samples 48 h after feeding. Figure 2-1 (lane 7) shows a positive PCR signal in *A. reductus* immediately after removal but a negative reaction in *A. reductus* starved 4 h (lane 8). The positive signal from *M. occidentalis* removed from prey for 24 h (lane 3) indicates *Wolbachia* is likely an intrinsic symbiont of this predator.

Sequence and Phylogenetic Analyses

Sequence information between the two primer sites was obtained for the eggs of *M. occidentalis* (COS strain) (849 bp), adult females of *M. occidentalis* (Russian Select strain) (850 bp), from 1 male *T. urticae* (840 bp) (GenBank accession numbers U44044, U44045, and U44046, respectively).

The *Wolbachia* sequences from the mites were similar to each other and to the *Wolbachia* from insects, but not to *Wolbachia persica* Suiter and Weiss from the fowl tick *Argas persicus* (Oken). Sequence similarities were calculated by aligning the sequences and dividing the number of similar

bases by the total number of bases. Previous estimates of sequence similarity (Johanowicz and Hoy, 1996) were underestimated due to the inclusion of missing or questionable DNA sequence information. There is 99.98% sequence similarity between the *Wolbachia* from the COS and Russian Select strains of *M. occidentalis*, 99.99% similarity between the *Wolbachia* sequences from the COS strain of *M. occidentalis* and its prey *T. urticae*, and 99.98% similarity between the *Wolbachia* sequences from the Russian Select strain of *M. occidentalis* and *T. urticae*. However, because the DNA was sequenced only once, the differences could be due to *Taq* polymerase errors, since the base differences were in conserved areas of the 16S rRNA gene.

Interestingly, the 16S rDNA from *M. occidentalis* and *T. urticae* were not more closely related to each other than they were to the type species *Wolbachia pipientis* from the northern house mosquito *Culex pipiens* L. There was 100% similarity between *W. pipientis* and *Wolbachia* from the COS strain of *M. occidentalis* eggs.

The molecular phylogeny (based on 625 bp from the 5' end of the gene) shows that the 3 mite sequences are within the insect *Wolbachia* clade, and in the same subgroup as the *Wolbachia* from *C. pipiens* (Figure 2-2). There were 396 informative characters used to determine the trees. There were 4 most parsimonious trees (treelength, 926; retention index, 0.76; consistency index, 0.73). The phylogenetic tree shown in Figure 2-2 is the consensus tree, constructed by collapsing the unresolved area of the tree into a region of soft polytomies (regions of ambiguous resolution) (Maddison, 1989) which is the subgroup containing the mite sequences. Thus, it is impossible to determine

which sequence in this subgroup is ancestral because of insufficient sequence information

Heat Treatment

I was unable to detect a *Wolbachia* specific PCR signal from a heat-treated population of the COS strain of *M. occidentalis* (Figure 2-1 lane 5), which is normally positive. DNA extracted at the same time from 60 eggs of a colony (COS) not subjected to the heat treatment was positive (Figure 2-1 lane 4), as expected. These results provide further evidence of an intrinsic infection in *M. occidentalis* since the *T. urticae* fed to these heat-treated mites were positive for *Wolbachia*.

Discussion

Based on the 16S rDNA analysis, the *Wolbachia* from *M. occidentalis* and *T. urticae* are related closely to *Wolbachia* from insects and not to *Wolbachia persica*, a microorganism found in the acarine *Argas persicus* (Suiter and Weiss, 1961). The primers were designed to specifically amplify *W. pipientis*, and because the 16S rDNA from *W. persica* and *W. pipientis* is so different (Weisburg *et al.*, 1991), it was expected that the mite sequences would be related to *Wolbachia* from insects. However, it was surprising that, although there has been a long isolation between the Chelicerata (acarines) and the Mandibulata (insects) of 550 million years (Manton, 1977), the *Wolbachia* 16S rDNA sequences from the 2 mite species are not more similar to each other than they are to that of the *Wolbachia* from the more distantly related insect *C.*

pipiens (Figure 2.2). While the *Wolbachia* from some closely related insect taxa grouped together, it is also the case that distantly related insects may have very similar *Wolbachia*. This lack of congruence between the evolutionary history of the arthropods and that of the *Wolbachia* suggests some horizontal transfer of *Wolbachia* between arthropod species may have occurred (Rousset *et al.*, 1992b; O'Neill *et al.*, 1992) in addition to vertical transmission. However, the mechanisms of such horizontal transfer have not been identified.

Wolbachia can be detected in otherwise *Wolbachia*-free *A. reductus* immediately after feeding on infected prey, therefore studies with predatory arthropods must take into account the infection status of their prey. Starving the mites for at least 4 h appears to eliminate false positive signals caused by transient infection. Based on a model of *M. occidentalis* feeding on *T. urticae* (Fransz, 1974), I calculated that 80% of the gut contents are digested within 4 h, which may be enough to bring transient *Wolbachia* DNA concentrations to undetectable levels. I found that starving the mites too long decreases the amount of *Wolbachia* detectable by the PCR, which is consistent with past studies in which the density of various symbionts from the citrus mealybug *Planococcus citri* (Risso) is decreased when they are starved (Iaccarino and Tremblay, 1970). Symbiont number is increased in the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles, after feeding (Burgdorfer *et al.*, 1973).

Although both predator and prey contain intrinsic *Wolbachia*, differentiation between the two species' *Wolbachia* based on 16S rDNA information was not possible. The 16S rDNA region sequenced is useful for some levels of phylogenetics (Weisburg *et al.*, 1989), but it does not allow for robust discrimination within the *Wolbachia* clade (O'Neill *et al.*, 1992).

Analysis of the entire 16S rRNA gene or of other more variable genes may have allowed a better discrimination between *Wolbachia* from *M. occidentalis* *T. urticae*, and insects. Sequence information from multiple clones of the 16S rDNA PCR products or from both strands of the PCR product may have increased the accuracy of the estimates of the differences between the *Wolbachia* from *M. occidentalis*, *T. urticae*, and insects. However a more accurate estimate of these differences is unlikely to change the conclusions of this study because this region is so conserved (O'Neill *et al.* 1992) and thus does not allow for fine scale analysis of *Wolbachia* diversity (Werren, 1997).

Although all laboratory population of *M. occidentalis*, except one, were positive for *Wolbachia* by using a PCR assay, the field-collected population was not. It is possible that other field populations of *M. occidentalis* may be infected, but simply were not sampled. *M. occidentalis* populations in California almond orchards, pear orchards, and vineyards have been shown to vary in pesticide resistances (Hoy, 1985), and thus may represent partially isolated populations caused by a relatively low rate of dispersal. It also has been demonstrated that some subpopulations of *Drosophila simulans* are infected whereas others are not (Hoffmann *et al.* 1986). High temperatures or naturally occurring antibiotics are possible reasons for these polymorphic populations (Hoffmann *et al.* 1990).

Although *Wolbachia* has been confirmed in several populations of *M. occidentalis* and different populations of *M. occidentalis* are known to exhibit partial nonreciprocal mating incompatibilities, there is no direct evidence yet that the incompatibilities actually are caused by the *Wolbachia*. This information would be particularly interesting because of proposals to use

Wolbachia as a drive mechanism to insert a desired trait into a population (Beard *et al* , 1993) Postmating incompatibilities caused by *Wolbachia* may aid in releases of genetically improved strains in biological control programs (Caprio and Hoy, 1995), once more is learned about the role Impact, and spread of *Wolbachia* in arthropods, and especially in *M occidentalis*

Table 2.1 *Wolbachia* infection status of mite populations

Species / Strain	Source [a]	Positive (+) or Negative (-) PCR Results egg	adult
PREDATORS			
Amblyseius reductus	A	–	
Metaseiulus occidentalis			
COS [b]	A	+	+
Russian Select[c]	A	+	+
Supermite	A	–	–
Hybrid Select	A	nt[e]	+
Ave-21	A	nt	+
Pullman Blackberry	A	+	+
WA Select	A	nt	+
Oregon Lab	B	nt	+
Visalia	C	nt	+
Griggs Apple	D		+
PREY			
Tetranychus urticae			
Florida laboratory[d]	A	+	+
Oregon laboratory	B	nt	+
Ohio laboratory	F	nt	+
Visalia insectary	C	nt	+
cotton field	E	nt	
Tetranychus turkestani	E	nt	+
Panonychus ulmi	D	nt	
Heat treated *M occidentalis*	A	–	–

[a] A= lab colony, Gainesville, FL B= lab colony, Corvallis, OR, C= insectary Visalia CA D= field, apple E= field, cotton, F= lab colony, Columbus, OH

[b] eggs sequenced

[c] adult female sequenced

[d] adult male sequenced

[e] nt =not tested

Figure 2.1 Bands of the expected size 900 bp were amplified using *Wolbachia*-specific 16S rDNA primers. Lane 1, Molecular weight marker VI (Boehringer Mannheim, Germany); lane 2, *M. occidentalis* adult females immediately after feeding (frozen before extractions); lane 3, *M. occidentalis* females starved 24 h; lane 4, *M. occidentalis* eggs; lane 5, heat treated *M. occidentalis* eggs; lane 6, *T. urticae* eggs; lane 7, *A. reductus* females fed spider mites tested immediately after feeding; lane 8, *A. reductus* females fed spider mites and starved for 4 h; lane 9, *D. simulans* positive control. Other nonspecific PCR products of much lower and higher molecular weights than expected using *Wolbachia* specific 16S primers were present in some samples.

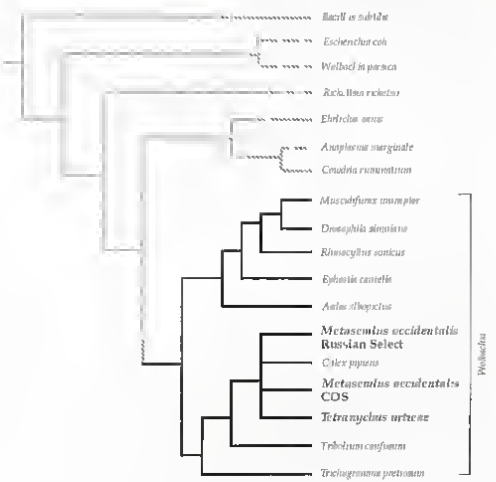


Figure 2-2. Phylogenetic tree of the 16S rDNA 5 region (*E coli* positions 100-773) from various microorganisms by using parsimony analysis (informative characters, 396, treelength 926 retention index 0 76 consistency index 0 73) The entire *Wolbachia* clade is drawn with solid lines Pictured is a consensus tree the branching pattern within the *Wolbachia* subgroup containing the 3 mite species Indicates soft polytomies in this region

CHAPTER 3
FURTHER GENETIC CHARACTERIZATION OF *WOLBACHIA* FROM *METASEIULUS OCCIDENTALIS* AND *TETRANYCHUS URTICAE* USING PARTIAL *FTSZ* GENE SEQUENCES

Introduction

Previous studies indicated *Wolbachia* endosymbionts are present in both the predatory mite *Metaseiulus* (=*Galendromus* *Typhlodromus*) *occidentalis* (Nesbitt) and its spider mite prey *Tetranychus urticae* Koch (Johanowicz and Hoy 1996 Chapter 2) A high degree of similarity between the *Wolbachia* 16S ribosomal DNA sequences from *M occidentalis* and *T urticae* (Johanowicz and Hoy 1996, Chapter 2) make it difficult to discriminate between the two types of *Wolbachia* in phylogenetic analyses Additionally, PCR-based assays of predators using 16S primers are difficult to interpret because DNA extractions of adult predators may contain spider mite *Wolbachia* DNA as a gut contaminant (Chapter 2)

The *Wolbachia ftsZ* gene important in prokaryotic cell division (deBoer *et al* , 1990), was unexpectedly discovered during screening of a *Drosophila melanogaster* genomic library (Holden *et al* 1993) The *ftsZ* gene is reported to be more variable than the 16S rRNA gene and therefore may have more power to discriminate between *Wolbachia* strains (Werren *et al* , 1995b)

The purpose of this chapter was to amplify part of the *ftsZ* gene and to sequence it to further characterize the *Wolbachia* from both predator and prey

mites If the sequence information from the *ftsZ* genes indicates variability exists between the predator and prey mite *Wolbachia* PCR primers will be designed that specifically amplify only one of the two different *Wolbachia* This would facilitate the PCR based detection of *Wolbachia* in individual predators by allowing DNA extractions to occur from a single, unstarved adult predator rather than from a starved adult or groups of eggs

Methods

Mite Maintenance and Sources

Metaseiulus occidentalis were maintained at the University of Florida and reared as previously described (Roush and Hoy 1981, Hoy *et al* 1982) *Tetranychus urticae* were raised on pinto bean *Phaseolus vulgaris*. plants in a greenhouse at the University of Florida-Gainesville

ftsZ PCR Primers

Primers were designed which amplify the *Wolbachia* bacterial septation gene *ftsZ* Preliminary PCR tests using two sets of primers designed by Werren *et al* (1995b) did not amplify *Wolbachia* DNA from either *M occidentalis* or *T urticae* Two new primers were designed from conserved regions of the *Drosophila melanogaster Wolbachia ftsZ* gene obtained from Genbank to amplify 310 bp of the gene from *T urticae* The primers used to amplify *Wolbachia ftsZ* DNA from T urticae are *forward ftsZ* 5' AAA CCG TTC GGT TTT GAA GGT GTG CGC CGT AT and *reverse ftsZ* 5'-GCA CTA

ATT GCT CTA TCT TCT CCT TCT GCC The expected size of the PCR product was approximately 310 base pairs (bp)

Two different new primers were later designed to amplify a portion of the *ftsZ* gene from *M occidentalis* These two new primers were designed from conserved areas outside the region that was amplified and cloned from *T urticae* This was done to reduce the risk of accidentally amplifying contaminating plasmid DNA from the cloned *ftsZ* gene fragments The primers for the *M occidentalis* experiment were designed to be specific to the B-group of *Wolbachia* because 16S rDNA analyses placed the *Wolbachia* in *M occidentalis* and *T urticae* in that group (Johanowicz and Hoy, 1996 Chapter 2) Potential primers were designed by aligning *Wolbachia ftsZ* sequences from 16 species obtained from Genbank and choosing 30 bp regions conserved only in the B-group *Wolbachia* The best primers from those conserved regions were chosen based on minimizing their potential secondary structure predicted by using MacDNASIS software (Hitachi Software) Secondary structure can interfere with priming efficiency (Saiki, 1989) The primers used to amplify the *Wolbachia* from *M occidentalis* are *ftsZf1* 5-TAC TGA CTG TTG GAG TTG TAA CTA AGC CGT, and *ftsZr1* 5-TGC CAG TTG CAA GAA CAG AAA CTC TAA CTC The expected size of the PCR product was approximately 570 bp The 570 bp fragment included the complete 310 bp region from *T urticae*

Polymerase Chain Reaction

M occidentalis DNA was extracted in Chelex from 10 pooled females starved at least 8 hours prior to extraction to avoid amplifying spider mite

Wolbachia present as contaminants in the guts of these predators (Johanowicz and Hoy, 1996 Chapter 2) *T urticae* DNA was extracted from individual females in Chelex as previously described and the PCR product from three individuals was later pooled for cloning

Cycling conditions were as follows 1 µl template DNA 50 mM KCl, 10 mM Tris HCl 1 5 mM MgCl2 0 2 µM each primer (both the forward and reverse primers according to mite species), 200 µM each dNTP and 0 8 units of *Taq* polymerase in a total volume of 25 µl Reactions were cycled 35 times at 94° C for 30 sec, and 72°C for 60 sec Because the primers were at least 30 bp long their theoretical T_m was large enough so that a two-step PCR reaction using a 72° annealing and extension temperature was possible

Cloning and Sequencing

The 310 bp fragment amplified and pooled from *T urticae* was cloned (T A overhang method Mead *et al* 1991) for subsequent sequencing Four clones were sequenced Three were identical except for one with 4 base substitutions These base differences were not present in any other *Wolbachia* sequences published to date These base differences produced a restriction enzyme recognition site specific to the enzyme AciI Restriction digests by this enzyme of the remaining 40 clones did not indicate the site was present in any others so it was not used in the analysis The two 30 bp regions corresponding to the priming sites were excluded from analysis If slight mismatches between the template and primers were initially present the primers might still work, and the PCR product would reflect the primer

sequences rather than the original template sequences. This left 261 bp for subsequent analyses.

The 570 bp fragment amplified from the pooled sample of *M. occidentalis* was cloned (T-A overhang method, Mead *et al.* 1991) for subsequent sequencing. Four clones were sequenced. All four were identical except for 1 or 2 non-synonymous base differences in three of the clones, presumably due to *Taq* polymerase errors. These base differences were not present in any other *Wolbachia* sequences published to date and appeared in highly conserved areas of the *ftsZ* gene, so these clones were not used in subsequent analyses. The two 30 bp regions corresponding to the priming sites were excluded from analysis. This left 509 bp for subsequent analyses.

Sequence Analysis

Sequences were aligned with MacDNASIS. The sequence from *T. urticae* was compared to the sequence from *M. occidentalis* on the basis of sequence similarity. The 509 bp *Wolbachia ftsZ* sequence from *M. occidentalis* was used in subsequent parsimony analysis with an unreleased version of PAUP (with permission from the author, Swofford 1997). With 26 *Wolbachia* sequences obtained from Genbank, PAUP's heuristic search algorithm with 100 bootstrap replicates (a resampling technique) was used to find the most parsimonious tree. This search method is appropriate when calculating phylogenies with large data sets (Swofford *et al.* 1996). A 50% consensus tree was calculated, meaning that the branching patterns which indicate an ancestry supported by at least 50% of the bootstrap replicates are kept as branches in the consensus tree. The unresolved areas, which do not support

any ancestral pattern due to insufficient sequence information, are collapsed into unbranched regions called 'soft polytomies' (Maddison 1989). No appropriately related *ftsZ* sequences were available (for example, other rickettsia) as an outgroup, which helps the computer program calculate the phylogeny more accurately (Swofford *et al.* 1996). Therefore, I used a technique called 'midpoint rooting', which is an appropriate solution when no reasonable outgroups exist (Swofford *et al.*, 1996).

Results

The 261 bp region of the *ftsZ* gene amplified from *T. urticae* was identical to the same region of the *ftsZ* gene amplified and sequenced from *M. occidentalis* (Figure 3-1).

Parsimony analysis indicated the 509 bp sequence from *M. occidentalis* grouped within the B-group *Wolbachia*. The *M. occidentalis* sequence was near the sequence obtained from the house mosquito *Culex pipiens* L., as it was when the 16S ribosomal DNA analysis was conducted (Johanowicz and Hoy, 1996, Chapter 2). The *ftsZ* gene from *M. occidentalis* was 98.4% similar to the *Wolbachia* sequence from *C. pipiens* L., corresponding to 8 bp differences (16S rDNA studies indicated they were 100% similar) and was 99.4% similar to the sequence from the grain moth *Ephestia cautella* (Walker), corresponding to 3 bp differences (16S rDNA studies indicated a 99% similarity).

The 50% consensus tree shows the *ftsZ* sequence of *Wolbachia* from *M. occidentalis* is located in an unresolved area (Figure 3-1). Out of 509 characters (nucleotide bases), 62 were determined by PAUP to be 'parsimony

informative meaning they provided the appropriate amount of variability to be useful in calculations of the phylogenetic estimates The other bases were either completely identical in all taxa (447 bases) or displayed too much variation (16 bases) to be useful in the analysis The tree-length was 129, which is a summation of the least number of base changes needed to explain the variations in DNA sequences. The consistency index was 0 64 and the retention index was 0 89 These indices give the relationship between the number of conceivable and observed base changes in the data set and the closer the value is to one, the better the tree predicts the most likely evolutionary pattern (Maddison and Maddison, 1992)

Discussion

The 261 bp fragment of the *Wolbachia ftsZ* gene obtained from both the predator and prey mites were identical. This ruled out the possibility of designing species-specific *Wolbachia* PCR primers for use on unstarved predator adults based on this sequence information

The 509 bp portion of the *Wolbachia ftsZ* gene used to compare *M occidentalis* with other arthropods did not provide more resolving power than the sequence information from the 16S rRNA gene (Figure 2-2 in Chapter 2) The 509 bp from the predator *ftsZ* sequence used in this phylogenetic analysis left regions of unresolved ambiguity in the subgroup where the *M occidentalis Wolbachia* sequence was positioned, just as in the earlier 16S rDNA phylogeny

Because the sequences from various arthropods within this unresolved area are quite similar, no conclusions can be drawn regarding the possibility of a horizontal transfer of *Wolbachia* between the predator and prey mites Sequence information from the eggs of *M occidentalis* might have confirmed that the DNA amplified from the starved adults was only from their own *Wolbachia* However upon realizing that the *ftsZ* region used was so conserved, no further sequencing was conducted Sequence information from a more variable region of the *ftsZ* gene perhaps from part of the non coding region or a longer stretch of the coding region, may be needed to discriminate between the *Wolbachia* in *M occidentalis* and *T urticae* and assess the possibility of horizontal transfer of the symbiont between these ecologically related species.

Taxonomically-distinct arthropods (Isopoda Acari Lepidoptera Orthoptera Coleoptera, and Diptera) host *Wolbachia* strains with similar *ftsZ* gene sequences as shown in Figure 3-2 Unless horizontal transfer of *Wolbachia* has occurred between distinct arthropod species the *Wolbachia* from these hosts are probably different strains with more genetic variability than can now be detected Efforts are under way to sequence other genes in *Wolbachia* (Werren, 1997) Sequence information from other, more variable genes might more accurately determine the similarity of *Wolbachia* obtained from various arthropod species and populations

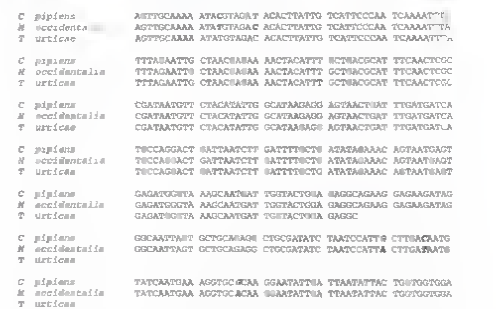


Figure 3 1 Sequence alignment of a partial *Wolbachia ftsZ* gene from *Culex pipiens* (the type species *Wolbachia*) *Metasemlus occidentalis* and *Tetranychus urticae* Bold italic print highlights the differences in nucleotide composition

Figure 3-2. Consensus phylogenetic tree constructed from 509 bp of the *Wolbachia ftsZ* gene. The sequence from *M. occidentalis* is situated in a region of unresolved ambiguity

CHAPTER 4
EXPERIMENTAL INDUCTION AND TERMINATION OF NONRECIPROCAL REPRODUCTIVE INCOMPATIBILITIES IN A PARAHAPLOID MITE

Introduction

Reproductive incompatibilities have been detected in various phytoseiid mites including *Metaseiulus* (=*Typhlodromus*; *Galendromus*) *occidentalis* (Nesbitt), a biological control agent of the two spotted spider mite *Tetranychus urticae* Koch. Associated with these intraspecific reproductive incompatibilities between different populations were shriveled eggs, low numbers of eggs, low survival of immature stages, and reduced fecundity in surviving F_1 individuals (Croft 1970, Hoy and Knop 1981, Hoy and Standow, 1982, Hoy and Cave 1988). Croft (1970) detected reciprocal reproductive incompatibilities (females from both of the two populations being crossed are incompatible with males from the different population) and nonreciprocal reproductive incompatibilities (females from only one of the two populations being crossed are incompatible with males from the other population) in crosses of *M. occidentalis* from California, Utah, and Washington. When Hoy and Knop (1981) crossed a laboratory selected permethrin resistant strain of *M. occidentalis* with its original base colony, few eggs were produced and many were shriveled and failed to develop in one cross while the reciprocal cross was compatible. They also found that a partially permethrin resistant strain was nonreciprocally incompatible with its base colony after only one

year of selections. Similar nonreciprocal reproductive incompatibilities were found in crosses with sulfur resistant *M. occidentalis* (Hoy and Standow 1982). In a later study, Hoy and Cave (1988) detected nonreciprocal partial reproductive incompatibilities between five colonies of *M. occidentalis*. Other examples of nonreciprocal incompatibilities in phytoseiids were found in *Typhlodromus annectens* DeLeon (McMurtry and Badii, 1989) and in two populations of *Amblyseius addoensis* van der Merwe and Ryke from South Africa (McMurtry, 1980).

Nonreciprocal reproductive incompatibilities are one of the effects associated with the presence of *Wolbachia* endosymbionts in a diverse array of Arthropoda, including insects (Mandibulata: Insecta), isopods (Mandibulata: Crustacea) and spider mites (Chelicerata: Arachnida) (reviewed by Werren, 1997). These small, fastidious rickettsia like symbionts are located intracellularly in the infected arthropods and are maternally-inherited through the egg cytoplasm. Infected females can successfully reproduce when crossed with infected or non-infected males, but crosses between uninfected females and infected males yield various phenotypes associated with the incompatibility, which varies depending upon the genetic system of the species. The incompatibilities are expressed as reduced numbers of viable progeny (both sexes) in diplo-diploid insects (Laven, 1951; Yen and Barr, 1974; Hoffmann *et al*., 1986; Hsiao and Hsiao, 1985; Wade and Stevens, 1985; Giordano *et al*., 1995), reduced numbers of diploid females in haplo-diploid insect parasitoids and an increased complement of haploid males (the females become haploid males) (Ryan and Saul, 1968; Breeuwer and Werren, 1990) and reduced numbers of females (with the normal complement of male

progeny) in some strains of the haplo-diploid spidec mite *Tetranychus urticae* (Vala and Breeuwer, 1996) The cytogenetic mechanism by which *Wolbachia* affects embryonic development and sex ratio appears to involve either a loss of the paternally derived chromosomes or aberrations in the paternal pronuclei early in embryonic development (Werren, 1997)

Rickettsia-like microorganisms were detected in the eggs and ovaries of the phytoseiid mite *M. occidentalis* by transmission electron microscopy (Hess and Hoy, 1982), which led Hoy and Cave (1988) to conclude that some of the incompatibilities seen in previous studies might be microorganism-mediated Molecular analyses with *Wolbachia*-specific 16S ribosomal DNA Polymerase Chain Reaction (PCR) primers indicated *Wolbachia* is present in many but not all, of the *M. occidentalis* populations examined (Johanowicz and Hoy 1996, Chapter 2) Phylogenetic analysis of the 16S ribosomal DNA (Johanowicz and Hoy 1996, Chapter 2) indicated the *Wolbachia* in *M. occidentalis* are genetically similar to the *Wolbachia* found in the insect *Culex pipiens* L. and in the spider mite *Tetranychus urticae* Although *Wolbachia* have been detected in mites (Johanowicz and Hoy, 1995, 1996 Chapter 2, Breeuwer and Jacobs, 1996, Tsagkarakou *et al* , 1996), relatively little is known about the biological effects of *Wolbachia* in parahaploid phytoseeds.

In insects (Arthropoda Mandibulata Insecta) and isopods (Arthropoda Mandibulata Crustacea), the effects of *Wolbachia* have been reversed with antibiotic or heat treatments (Yen and Barr, 1973 Richardson *et al* 1987 O'Neill, 1989 Breeuwec and Werren, 1990, Stouthamer *et al* 1990b Louis *et al*., 1993) Heat treatment also reduced the presence of *Wolbachia* to

undetectable levels in *M. occidentalis* (Arthropoda Chelicerata Arachnida) (Johanowicz and Hoy, 1996, Chapter 2)

Crosses between infected and uninfected individuals are a standard technique used in studies of *Wolbachia* in insects (Werren, 1997) Crosses between inbred laboratory populations of *M. occidentalis* differing in the presence or absence of *Wolbachia* due to heat treatment should allow a correlation between the presence of *Wolbachia* and nonreciprocal incompatibility Using inbred lines of *M. occidentalis* reduces the effects of nuclear genetic differences that might cause premating incompatibilities which could confound measures of *Wolbachia*-mediated cytoplasmic incompatibility

The objectives of this chapter were 1) determine whether uninfected *M. occidentalis* females are incompatible with males containing *Wolbachia*, 2) determine whether compatibility between the infected males and cured females can be restored if the males are later cured, and 3) correlate any incompatibilities with the presence of *Wolbachia*

Methods

Mite Maintenance and Sources

Metaseiulus occidentalis were maintained at the University of Florida and reared as previously described (Roush and Hoy 1981 Hoy *et al* , 1982) A colony of two spotted spider mites was raised on pinto bean *Phaseolus vulgaris* L., plants in a greenhouse at the University of Florida-Gainesville. An inbred, isofemale line of *M. occidentalis* was initiated three months prior to the

study by isolating one gravid female allowing her progeny to sib mate, removing one gravid female, and repeating this procedure for four generations at 24°C *M. occidentalis* is tolerant of inbreeding (Hoy, 1977 Bruce-Oliver and Hoy 1990) and this procedure allowed reduction of differences in the nuclear genome as factors in the subsequent experiments Two of the resulting gravid generation five (G5) females were used to initiate two new lines one line (RT) was maintained at normal rearing temperatures (24°C) and the second line was held at 33°C for at least 8 generations (HT) Temperatures >30°C administered for a few generations reduce or eliminate *Wolbachia* in some insects (Stevens 1989, Stouthamer *et al* 1990a, Girin and Louletreau, 1995 Louis *et al* , 1993)

A third colony (R-->H) was later initiated by removing 100 gravid females from the inbred RT line allowing them to mate *inter se* while maintaining them at 33°C for at least 10 generations Thus line was used to test whether compatibility could be restored between it and the original HT line

Experiment 1 Tests for Incompatibilities

Crosses were conducted on 4 2 cm^2 pinto bean leaf discs on water soaked cotton The leaf discs were infested with all stages of spider mites as prey Expenments were performed under constant light at 22-24°C and 45-65% RH *M. occidentalis* eggs or newly eclosed larvae were isolated on leaf discs with prey, allowed to mature to adults and sexed Single pairs of one- to two-day-old adult virgin females and males were introduced on the first of a series of four leaf discs where they were allowed to mate and deposit their first eggs. The females then were moved to new leaf discs daily for a total of

four days The location of the eggs was marked daily with India ink to make relocating them easier The number of shriveled eggs, surviving progeny and developmental stage of the progeny were recorded each day The progeny sex ratio was determined by recording the sex of adult progeny

Twelve single pair crosses (female x male) of each of the four crossing types were made (HT x RT RT x HT, HT x HT and RT x RT) (Figure 3-1), for a total of 48 crosses Females which never became gravid were excluded from analysis Crosses which did not yield adults were excluded from the sex ratio analysis Data were analyzed by one-way ANOVA and pairwise comparisons were made with Scheffe's procedure (StatView, Abacus Concepts 1992) at alpha <0.05

Experiment 2 Tests for Restored Compatibility

Methods were similar to those of the previous experiment except temperatures and relative humidities were between 23°-25°C and 50 70% RH Because it was difficult to differentiate between shriveled predator eggs and partially-consumed spider mite eggs (Figure 4-2), the number of shriveled *M accidentalis* eggs in the experiments may be underestimated

The subpopulation of RT mites subjected to heat-treatment (R-->H) were crossed with both the HT and RT lines to determine whether incompatibilities between HT females and RT males would disappear when substituting R-->H males, and whether new incompatibilities would appear between R-->H females and males from its (RT) base colony Twelve single pair crosses of each of the nine crossing types were made (HT x RT RT x HT RT x R-->H R-->H x RT, R-->H x HT, HT x R-->H, HT x HT, RT x RT and R--

>H x R-->H) (Figure 4-1) which resulted in a total of 108 single pair crosses Data were analyzed by one-way ANOVA and pairwise comparisons were made with Schaffe's procedure at alpha <0 05

Infection Status

The PCR was used before the first experiment to test the infection status of the RT and HT lines with *Wolbachia*-specific PCR primers which amplify the 16S ribosomal DNA gene PCR conditions were as previously described (Johanowicz and Hoy, 1996) DNA from five starved females was pooled for each PCR reaction in an effort to increase the amount of *Wolbachia* DNA and reduce false negatives due to possible low titers of this symbiont in these tiny (0 3 x 0 15 mm) mites Three PCR reactions each were performed on the RT and HT lines

Primers which amplify the *ftsZ* gene of *Wolbachia* were used to evaluate the infection status of individual females after the second experiment was completed Three primers were designed for a hemi nested amplification to increase sensitivity and to be specific to B-group *Wolbachia* because 16S rDNA analyses place the *Wolbachia* in *M occidentalis* in that group (Johanowicz and Hoy, 1996, Chapter 2) A-group specific *ftsZ* primers did not amplify DNA in *M occidentalis* (unpublished data) New primers were designed by aligning *Wolbachia ftsZ* sequences obtained from Genbank and choosing areas conserved in B-group *Wolbachia* The primers used were *fts*Zf1 5' TAC TGA CTG TTG GAG TTG TAA CTA AGC CGT *fts*Zf2, 5'-GGA GAA GAT AGG GCA ATT AGT GCT GCA GA and *fts*Zr1 5' TGC CAG TTG CAA GAA CAG AAA CTC TAA CTC

DNA was extracted from the eggs of single, isolated females by allowing adult females to lay eggs for 3 days, the resultant 5-7 eggs were collected pooled, and extracted in 25 µl Chelex (Johanowicz and Hoy, 1996) This procedure allowed estimates of the proportions of infected females without the risk of amplifying contaminating spider mite *Wolbachia* DNA from the digestive tract of the predators (Johanowicz and Hoy, 1996, Chapter 2) Eggs from 20 RT, 10 HT, and 10 R-->H females were tested

Cycling conditions for the first round of amplification were as follows 1 µl template DNA 50 mM KCl, 10 mM Tris HCl, 1.5 mM $MgCl_2$, 0 2 µM each primer (*fts*Zf1 and *fts*Zr1), 200 µM each dNTP and 0.8 units of *Taq* polymerase in a total volume of 25 µl Reactions were cycled 35 times at 94°C for 30 sec, and 72°C for 60 sec Cycling conditions for the second round of amplification were the same as above, except 1 µl of the previously amplified DNA was used as the template, and primers *fts*Zf2 and *fts*Zr1 were used, producing a PCR product of approximately 250 bp Reagent-negative controls were included in the reactions

Results

Induction of Incompatibility

Nonreciprocal reproductive incompatibility was induced in crosses between the HT (cured) females and RT (infected) males This cross resulted in reduced numbers of eggs/female/day (mean ± s d = 0 2 ± 0 2) compared to the reciprocal cross (2 0 ± 0 8), and the maternal (2 0 ± 0.4) and paternal control (1 7 ± 0.8) crosses Higher percentages of shriveled eggs (62.5% ± 51 7)

were produced compared to the reciprocal (1 4% ± 4.2), maternal (0%) and paternal control crosses (2 2% ± 7 0) (Table 4-1) Figure 4-2 illustrates the appearance of shriveled eggs. Only male progeny were produced in the incompatible crosses between HT females and RT males Both males and females were produced in the reciprocal (RT x HT) and control crosses (Table 4-1)

The sex ratio in the RT x RT (infected) control crosses was more male-biased (62% males Table 4-1) than expected (33% males) (Lee and Davis, 1968 Nagelkerke and Sabelis 1991) Though there is a difference in sex ratio there was not a statistically significant difference in the number of eggs/ female/ day between the infected control crosses (1 7 ± 0 8) and the uninfected control crosses (2.0 ± 0 4)

Restoration of Compatibility

Incompatibilities similar to those observed in the first experiment (skewed sex ratio, shriveled eggs, reduced numbers of progeny) were detected in the crosses of HT females x RT males (Table 4-2) New incompatibilities were induced in the R-->H female x RT male crosses as expected, with females producing reduced numbers of eggs/female/day (0.8 ± 0 4) increased proportions of shriveled eggs/female/day (72 9% ± 32 8) and no female progeny The reciprocal crosses (RT female x R-->H male) produced a mean of 1 8 ± 0.5 eggs/female/day, only 4 7% ± 6 1 of those eggs shriveled, and 52% of the adult progeny were females

R-->H (cured) females crossed with HT (cured) males and the reciprocal cross (HT female x R-->H male) were compatible as expected

(Table 4-2) The mean number of eggs/female/day in the crosses was 2.8 ± 0.2 and 2.6 ± 0.4, respectively, and the mean percentage of shriveled eggs was 2.8% ± 6.6 and 1.7% ± 3.7, respectively The compatibility of HT females with RT males was therefore restored in experiment two when the males were subsequently heat treated (R >H) (Table 4-2) These results indicate the incompatibilities are due to a heat sensitive cytoplasmic factor and are not due to nuclear genetic differences

As in experiment 1, the sex ratio in the RT x RT crosses was more male-biased (59% males) than expected (Table 4-2), so two crosses determined to be incompatible based on a compatibility index were excluded in an additional analysis The compatibility index was calculated as: (the number of viable eggs + number of daughters number of shriveled eggs) ÷ 10 Crosses were scored as compatible if the Compatibility Index was greater than 0.35 and incompatible (uninfected) when less than 0.35 The threshold value of 0.35 clearly separated the two types The appearance of an unexpected incompatible cross could be due to imperfect maternal transmission of the *Wolbachia* to a daughter, which is known to happen in insects (Turelli *et al.* 1992) The percentage of males initially calculated as 59.4 ± 22.3 changed to 50.4 ± 10.9 Thus male-biased sex ratio is still higher than expected but not significantly higher than the cured control crosses However as in the previous experiment (after eliminating the two unexpected "incompatible" crosses), the mean number of eggs/ female/ day in the infected (RT x RT) control crosses (2.06 ± 0.4) was not significantly different than in the HT x HT (2.36 ± 0.45) and R->H (2.35 ± 0.58) uninfected control crosses at alpha < 0.05

Infection Status

The initial PCR tests were in agreement with past studies (Johanowicz and Hoy, 1996 Chapter 2) All three pooled samples from mites held at 24°C (RT) were positive for *Wolbachia* by the PCR and the line reared at 33°C (HT) had undetectable amounts of *Wolbachia* in all three of the samples tested The PCR assay with *ftsZ* primers at the end of the second experiment indicated none of 10 HT females were positive for *Wolbachia* as expected, none of 10 R-->H females were positive as expected, and 12 of 20 RT females were positive Possible reasons why 8 of the 20 RT females were not positive include loss of the symbiont in some individuals over time due to laboratory rearing stresses (crowding and/or nutritional stresses affect *Wolbachia* density in *Drosophila simulans* (Sinkins *et al* , 1995a)) or low symbiont titers in the minute eggs of the individuals and subsequent failure of the PCR

Discussion

These experiments demonstrate that the temperature at which *M occidentalis* is reared can be used to induce or eliminate nonreciprocal reproductive incompatibility associated with the presence or absence of *Wolbachia* in *M occidentalis* Incompatibility was induced between HT (cured) females and RT (infected) males of an inbred line Compatibility between the two lines was subsequently restored when the males from the room-temperature line were heat treated (R-->H) and crossed with the HT females The nonreciprocal nature of the incompatibilities and the ability to restore

compatibility indicates a heat-sensitive cytoplasmic agent is responsible for the observed results. One cytoplasmic difference associated with the incompatibility is the presence or absence of *Wolbachia* endosymbionts as assayed by the PCR. The PCR results indicate *Wolbachia* is present in the RT line and eliminated in the HT mites. Although there could be some other unknown cytoplasmic factor responsible for the observed results, the data are consistent with what is currently known about the effects of *Wolbachia* on reproductive incompatibilities in insects.

Wolbachia-mediated incompatibilities in arthropods have various effects on progeny number and sex ratio based on the genetic system and the taxonomic group. *M. occidentalis* (Arachnida: Acari: Gamasida: Phytoseiidae) has a genetic system called parahaploidy (Hoy, 1979) which is sometimes termed pseudoarrhenotoky (Schulten, 1985). In parahaploidy, the embryos destined to become males are derived from fertilized eggs, but at the onset of the reductional division 24-48 hours after egg deposition, one set of chromosomes (most likely the paternal set) becomes heterochromatinized and excluded from the nucleus, producing a haploid male (Nelson-Rees *et al.*, 1980). Female embryos remain diploid. The incompatibility phenotype resulted in reduced progeny production, as in diplo-diploid insects (e.g. Insecta: Diptera, Coleoptera), and skewed, highly male-biased sex ratios, as in haplo-diploid insects (e.g. Insecta: Hymenoptera) and the mite *T. urticae* (Arachnida: Acari: Actinedida: Tetranychidae).

Because *Wolbachia*-mediated incompatibilities cause the destruction of the paternal set of chromosomes in insects (Werren, 1997), and because adult males rather than females were produced in some of the crosses, these crosses

may provide further evidence that the paternal set of chromosomes is the set which is eliminated during the embryonic development of these parahaploid mites (Hoy 1985). The reduced numbers of male progeny produced may be due to the effects of *Wolbachia* on the paternal set of chromosomes very early in development. Hoy (1979) found both the maternal and paternal sets are initially necessary for normal development in *M. occidentalis* males. The fertility of the few surviving male progeny is unknown.

The presence of incompatibilities like those due to *Wolbachia* infection have potentially interesting consequences for biological control programs. Some of the results are similar to those seen in previous hybridization studies where two phytoseiid populations were crossed to determine their species status (Croft, 1970, McMurtry *et al.*, 1976, McMurtry 1980, McMurtry and Badii, 1989). It may be important to consider whether *Wolbachia* mediated or heat induced incompatibilities occur when crossing mites from different origins or environmental conditions. Variations in presence, absence or density of this symbiont have been detected in field populations of insects, perhaps due to naturally-occurring antibiotics (Hoffmann *et al.*, 1990), high temperatures or diapause (Perrot-Minnot *et al.*, 1996). *Wolbachia* density may affect expression of incompatibility in some insects (Breeuwer and Werren 1993, Sinkins *et al.* 1995a). Two PCR surveys for *Wolbachia* in phytoseiids found both infected and uninfected populations in the field and in the laboratory (Breeuwer and Jacobs, 1996, Johanowicz and Hoy, 1996, Chapter 2), which could account for some of the previous reports of nonreciprocal incompatibilities in crosses between phytoseiid populations.

Despite extensive inbreeding for these experiments, the reproductive parameters (egg production, immature mortality) measured in the parental crosses appear normal. Previous studies by Hoy (1977) also found that this species is tolerant of inbreeding.

There was, however, a slight male-bias in the sex ratio of the RT parental control crosses (Tables 4-1 and 4-2). This could be due to the inclusion of a few unexpected incompatible control crosses in the analysis. Imperfect maternal transmission of the symbiont to the females used in the incompatible crosses may be responsible for the unexpected incompatibilities, as has been demonstrated in *Drosophila simulans* Sturtevant (Turelli *et al.* 1992). After removing those RT control crosses determined to be incompatible, the bias did not decline in the first experiment (both nearly 62% males), but it did decline in the second experiment, from approximately 60% males to slightly more than 50% males, which is still higher than expected. Following theoretical predictions by Hamilton (1967) and Nunney (1985) based on inbreeding potential and local mate competition, sex ratios should be female-biased as an adaptive response to low foundress density in a subdivided population structure. A subdivided population structure is common in phytoseuds which specialize in patchily distributed spider mites (Sabelis and Nagelkerke, 1993). Therefore, a female biased sex ratio is expected to occur in *M. occidentalis*. The sex ratio of *M. occidentalis* can be as high as 50% male in the laboratory (Lee and Davis, 1968), but is usually female-biased, with approximately 33% males (Bruce-Oliver and Hoy, 1990, Tanigoshi *et al.* 1975). Nagelkerke and Sabelis (1991) found similar female-

biased sex ratios of *M. occidentalis* when the mothers were isolated on their own leaf arenas.

The male-bias declined (60% -> 35%) after the KT line was subjected to heat (K-->H). This indicated a nuclear genetic component was not responsible for the bias. In addition, the bias was not due to the mothers having "precise control" of their progeny sex ratio as a response to prey and conspecific density, as observed by Nagelkerke and Sabelis (1991), because all of these crosses had similar prey density and other environmental conditions. Rather, a heat-sensitive cytoplasmic factor, most likely *Wolbachia*, is responsible for the reduced production of daughters in the infected mites. Fecundity losses associated with *Wolbachia* infection have been detected in insects (Hoffmann and Turelli, 1988), and these results suggest that in addition to the induction of nonreciprocal reproductive incompatibility, there also may be negative fitness costs due to *Wolbachia* infection in *M. occidentalis*.

Table 4-1 Experimental induction and termination of *Wolbachia* mediated incompatibility in an inbred line of *M. occidentalis* by heat-treatment
One sub-population was reared normally at 25°C (RT) and one was reared at 33°C (HT) Same letters in a column following means are not significantly different at alpha <0 05 using Scheffe s procedure

Cross Type Female x Male	No crosses producing eggs/ total no crosses	Mean no eggs /day (s d.)	Mean % eggs shriveled (s d)	Mean % immature deaths (s.d)	No crosses producing adults	Mean no. female progeny /cross (s.d)	Mean no male progeny /cross (s.d)	Mean % males
Experimental Crosses								
HTxRT*	8/11	0.2a (0 2)	62.5a (51 7)	0a -	3	0a —	1.0a —	100.0a —
RTxHT	9/10	2.0b (0.8)	1 4b (4.2)	5.9ab (9.6)	9	4 1b (1.4)	3 7b (1 4)	47 8bc (13.4)
Control Crosses								
HTxHT	12/12	2 0b (0.4)	0a	5.2ab (11.3)	12	4.6b (1 7)	2.7ab (1.6)	35.4b (20.6)
RTxRT	10/12	1.7b (0 8)	2.2b (7 03)	16.5b (14.11)	10	2.6ab (1.5)	3 8b (0.8)	61 6c (17 1)
F value		22.2**	14.5**	4.2		9 1**	4.9**	12.7**

* expect incompatibilities
** one-way analysis of variance significantly different p<0 01

Table 4-2 Compatibility can be restored and new incompatibility can be induced by heat-treatment of *M. accidentalis*

Cross Type Female x Male	No crosses producing eggs/ total no crosses	Mean no eggs/ day (s.d.)	Mean % eggs shriveled (s.d.)	Mean % immature deaths (s.d.)	No crosses producing adults	Mean no female progeny /cross (s.d.)	Mean no male progeny /cross (s.d.)	Mean % males
Experimental Crosses								
HTxRT[a]	10/11	0.9* (0.4)	91.7* (18.0)	0 -	2	0* -	1.5 (0.7)	100.0*
RTxHT	12/12	2.0* (0.6)	15.9* (26.8)	17.2 (20.4)	12	3.3* (2.0)	2.1 (1.6)	40.0* (30.9)
RTxR-H	10/10	1.8* (0.5)	4.7* (6.1)	7.0 (13.4)	10	3.4* (1.6)	2.9 (0.9)	48.2* (11.5)
R-HxRT[a]	12/12	0.8* (0.4)	72.9* (32.8)	2.1 (7.2)	6	0* 0	1.3 (0.5)	100.0*
R HxHT	10/10	2.8 (0.2)	2.8 (6.6)	13.2 (12.0)	10	5.4 (2.3)	4.0 (1.5)	44.4 (19.2)
HTxR-H[b]	11/11	2.6 (0.4)	1.7 (3.7)	7.7 (8.5)	11	5.5 (1.5)	4.0 (1.5)	42.0 (11.5)
Control Crosses								
HTxHT	12/12	2.4 (0.5)	0.8 (7.9)	10.6 (15.7)	12	4.5 (1.5)	3.8 (1.4)	45.4 (9.8)
RTxRT	11/11	1.9 (0.6)	7.7 (11.5)	12.0 (13.0)	11	2.7 (1.7)	3.2 (0.9)	59.4 (22.3)
R-HxR H	12/12	2.4 (0.7)	0.00 -	20.5 (30.1)	11	5.5 (2.2)	2.9 (1.0)	37.6 (22.3)
F value		23.8**	47.9**	1.9		9.2**	4.7**	8.7**

* following mean indicates significant differences between reciprocal crosses using Scheffe's procedure at alpha <0.05

a/ expect incompatibilities

b/ expect restoration of compatibility

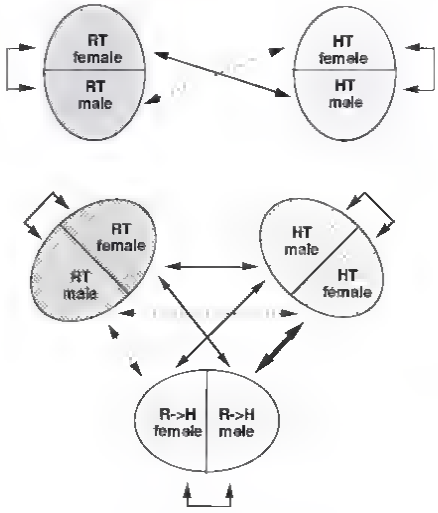


Figure 4-1 Pattern of compatibility between populations ←----→ indicate crosses expected to be incompatible ←——→ indicate crosses expected to regain compatibility and ←——→ indicate compatible crosses RT= mites reared at room temperature (infected), HT = cured mites, R-->H = cured mites

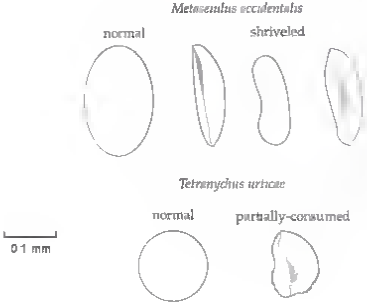


Figure 4-2. Normal and shriveled eggs of *M. occidentalis*. Normal *T. urticae* eggs and shriveled eggs after being partially consumed by *M. occidentalis*

CHAPTER 5
WOLBACHIA INFECTION DYNAMICS IN EXPERIMENTAL LABORATORY POPULATIONS OF *METASEIULUS OCCIDENTALIS*

Introduction

Wolbachia symbionts are responsible for numerous reproductive alterations in arthropods including nonreciprocal reproductive incompatibilities between uninfected females and infected males (reviewed by Werren 1997). When both infected and uninfected individuals are present in a population these nonreciprocal incompatibilities translate into a selective advantage to infected females (Caspari and Watson, 1959; Turelli and Hoffmann, 1991). This is because infected females can reproduce normally with any male they encounter, while uninfected females mated with infected males produce few or no progeny.

Since *Wolbachia* is transovarially transmitted, the reproductive advantage of infected females theoretically acts to rapidly increase the prevalence of *Wolbachia* infected hosts in a population (Caspari and Watson 1959; Fine 1978; Hurst, 1991; Stevens and Wade, 1990; Hoffmann *et al.* 1990; Turelli and Hoffmann 1991). Caspari and Watson (1959) developed a set of theoretical, analytical models describing the dynamics and equilibria of the "incompatibility factor", now known to be *Wolbachia*. They assumed total incompatibility between the proportion of males infected (designated by the letter "*i*") and the proportion of uninfected females ("*h*"), panmixis, a 1:1 sex

ratio, complete maternal transfer and a fecundity benefit (*S*) was associated with the uninfected type (that is a fecundity cost to those infected). Their equation to predict the prevalence of *b* (uninfected) females in the following generation *b'* is written as

$$b' = \frac{Sb^2}{Sb^2 + ab + a^2}$$

which can be rewritten using the more common terms to describe the Hardy-Weinberg law (Roughgarden, 1979). Here p_t = proportion of infected individuals at time *t*, $q_t = 1 - p_t$ = proportion of uninfected individuals at time *t* and w_i is the fitness cost associated with infection ($w_i < 1$), and is written as

$$p_{t+1} = \frac{w_i p_t^2 + w_i p_t q_t}{w_i p_t^2 + w_i p_t q_t + q_t^2} \quad \text{and} \quad p + q = 1 \qquad (1)$$

Note the lack of a "$2pq$" term in the denominator to describe the frequency of the "hybrids" in a population as is the case in Hardy-Weinberg equilibria when both hybrids are the same. In the case of *Wolbachia* induced incompatibilities one of the hybrids is inviable, and the other hybrid carries the *Wolbachia*.

Caspari and Watson's model predicts that if there is a fitness cost to infection then using the form in equation (1) there are three equilibrium points (p^*) for p, the frequency of *Wolbachia* infection. These are $p^* = (0, 1-w, 1)$. Zero and one are stable attracting points with the unstable threshold point $1-w$ in between. If we start with p below $1-w$, then the system is attracted to zero frequency of *Wolbachia* infection, if we start with p above $1-w$, the system is attracted to fixation of *Wolbachia* at frequency one.

Models by Fine (1979) and Hoffmann *et al.* (1990) expand on Caspari and Watson's initial work with algebraically similar models but include three

important parameters which affect the stable and unstable equilibria One parameter is the proportional failure of a mother to transmit *Wolbachia* to her offspring (imperfect maternal transmission, μ) The second is the proportion of progeny produced by the incompatible crosses relative to the compatible crosses, called 'hatchability' (H) The third is the relative fitness of infected matings relative to uninfected matings (F), usually measured in terms of productivity (Hoffmann *et al* 1990, Turelli and Hoffmann 1995) In these models, one stable point is zero, and the other moves away from fixation so both infected and uninfected individuals can coexist in a population if there is imperfect maternal transmission The models by Fine (1979) and Hoffmann *et al* (1990) also predict unstable frequencies which would prevent *Wolbachia* from spreading through a population if it is sufficiently rare The more elaborate model by Hoffmann *et al* (1990), which takes into account a modified dynamical behavior due to inclusion of the additional parameters predicts that

$$\hat{p}_{t+1} = \frac{p_t(1-\mu)(1-s_f)}{1-s_f p_t - s_h p_t(1-p_t) - \mu s_h p_t^2(1-s_f)} \qquad (2)$$

where

$s_f = 1 - F$ $s_h = 1 - H$, and p_t = proportion of infected individuals The equations describing the stable (p_s) and unstable equilibria (p_u) are therefore

$$p_s = \frac{s_f + s_h + \sqrt{(s_f + s_h)^2 - 4(s_f + \mu F)s_h(1-\mu F)}}{2s_h(1-\mu F)} \qquad (3)$$

and

$$p_u = \frac{s_f + s_h - \sqrt{(s_f + s_h)^2 - 4(s_f + \mu F)s_h(1-\mu F)}}{2s_h(1-\mu F)} \qquad (4)$$

Again, the unstable frequencies are sensitive to the fitness costs of infection (Turelli and Hoffmann, 1995)

Laboratory studies have documented the "spread" of *Wolbachia* in population cage studies (Hoffmann *et al* 1990 Sinkins *et al* 1995b) When an intermediate proportion of infected and uninfected *D simulans* were placed in population cages, the proportion of *Wolbachia*-infected individuals increased rapidly within 5-10 generations to approximately 80-95% (Hoffmann *et al*., 1990) The spread of *Wolbachia* was also "accidentally" discovered in a study by Sinkins *et al* (1995b) After microinjecting *Wolbachia* infected *D simulans* with a new strain of *Wolbachia* they found that only 10% of the individuals in a population harbored the double infection They subsequently monitored the fate of this double infection and found it increased from an initial prevalence of 10% to over 90% in only 12 generations (Sinkins *et al*., 1995b)

Field studies have documented the increases of individuals infected with *Wolbachia* in natural *D simulans* populations in California (Turelli and Hoffmann, 1991, 1995) These authors found that the proportion of infected individuals, first discovered in Southern California has increased within various local populations to a stable frequency of approximately 0 94 and the proportion appears to be increasing to that level in other local populations This stable equilibrium of 0 94 is similar to the frequency predicted by the theoretical models using the appropriate parameter values In addition to the spread of infections within local populations, the infection is also spreading northward at approximately 100 km per year (Turelli and Hoffmann, 1991

Turelli *et al* 1992) Field studies indicated that one particular mitochondrial variant is spreading along with the *Wolbachia* by "hitchhiking" with the infected cytotype (Turelli *et al*, 1992)

Several studies are in progress to genetically engineer and improve arthropods, for example, to be refractory to disease agents such as malaria parasites (Beard *et al* 1993) However, the success of any genetic control strategy that uses transgenes will depend on a mechanism which will favor the spread of the introduced genes through natural populations (Evans, 1993) The ability of *Wolbachia* and its associated cytoplasmic elements, like mitochondria or other symbionts, to spread through a population might be harnessed as a mechanism to "drive" desired traits through wild-type natural populations. This could happen if the transgene "hitchhikes" with the infected cytoplasm (Curtis, 1992, Beard *et al* 1993) However, the dynamics of *Wolbachia* are complex (Turelli, 1994 Prout, 1994), so data on the ability of *Wolbachia* to spread through populations are necessary to evaluate the feasibility of this mechanism in various arthropods.

Previous studies (Chapter 4) indicate *Wolbachia* in the predatory mite *Metaseiulus occidentalis* is associated with strong nonreciprocal reproductive incompatibilities between infected males and uninfected females *M occidentalis* has traditionally been used in ecological studies because of its rapid generation time and ease of rearing (e g Huffaker, 1958) and it is a subject of genetic improvement programs (Hoy, 1985 1994) For these reasons I chose to conduct an experiment to evaluate the potential of *Wolbachia* to spread through experimental laboratory populations of *M occidentalis*

This chapter reports the dynamics of *Wolbachia* infection in polymorphic laboratory populations of *M. occidentalis* over 12 generations with a low initial infection frequency of 0.1. This low initial infection frequency was chosen because it is likely to provide an appropriate test of the theory under realistic situations. Because of difficulties in estimating absolute population densities (Proverbs 1974, Caprio *et al.*, 1991), and in mass rearing high quality arthropods inexpensively (Bush 1979, Marroquin 1985, Mueller-Beilschmidt and Hoy, 1987, Hoy *et al.*, 1991, Hoy, 1994), the number of *Wolbachia*-infected arthropods released as part of any arthropod management project may not be more than 10% of the wild type population in a given area.

Methods

Mite Maintenance and Sources

Metaseiulus occidentalis were maintained at the University of Florida and reared as previously described (Roush and Hoy, 1981, Hoy *et al.*, 1982). The two-spotted spider mites, *Tetranychus urticae* Koch, were raised on pinto bean, *Phaseolus vulgaris* L., plants in a greenhouse at the University of Florida-Gainesville. Genetically similar infected and heat-cured *M. occidentalis* were used for the experiments. They were initiated one year prior to this experiment, initially for the experiments in Chapter 4, by isolating one gravid female, allowing her progeny to sib mate, removing one gravid female, and repeating this procedure for four generations at 24°C. Two of the resulting gravid generation five (G5) females were used to initiate two new lines, one line (RT) was maintained at normal rearing temperatures (24°C) and the

second line was held at 33°C (HT) The presence of *Wolbachia* in at least 80% of the individuals from the RT line and its absence in the HT line was confirmed by PCR of the *ftsZ* gene (Chapter 4) two months prior to this study

Three replicate populations of the RT and HT lines were initiated at the start of this study by moving 100 eggs to new population cages These populations were designated as RT- and HT- 1, 2, and 3 A third line was also initiated in triplicate for this study to assess the stability of heat-curing when the heat-treated mites are kept at normal rearing temperatures (H->R) In addition to these three control conditions, the "mixed" (polymorphic) experimental populations were initiated in triplicate with ten eggs from the infected RT population and 90 eggs from the HT (cured) populations

The RT, H >R, and mixed populations remained at normal rearing temperatures for the duration of this experiment Each population was subcultured every 3 weeks (to reduce crowding and fungal contamination) by moving 125 randomly-selected gravid females (of approximately 400) to a new population arena The food sources of the predators were monitored to avoid contamination of the populations In addition the population arenas were housed separately in polypropylene boxes, lined on the inside with a thick, 4 cm-wide band of petroleum jelly to discourage movement in and out of the boxes.

Progeny Testing for Compatibility

An assay method called "progeny testing" has been used to estimate the proportions of infected individuals in a population (Hoffmann *et al*, 1990) These assays are conducted by introducing infected males to females of

unknown *Wolbachia* status, and the number of compatible test crosses is used to estimate the proportion of females infected with *Wolbachia*

Alternatively, a Polymerase Chain Reaction (PCR) assay for *Wolbachia* infection can be used (O'Neill *et al* , 1992, Werren *et al* , 1995, Turelli and Hoffmann, 1995) A PCR-based assay has been demonstrated to be simpler and equally effective in predicting infection status in *D simulans* (Turelli and Hoffmann 1995) In *M occidentalis*, a PCR based assay can be problematic because of their small size and the possibility of false-positive signals from their diet of *Wolbachia*-infected spider mites (Johanowicz and Hoy, 1996 Chapter 2) A PCR- based assay for *Wolbachia* necessitates starving the mites before DNA extraction or collecting and combining eggs from individual females for subsequent DNA extractions and hemi nested PCR reactions (Chapter 3) Previous studies also have indicated that the reliability of the PCR assay on these eggs may be questionable (Chapter 4) and that storage of DNA extractions reduced the sensitivity of the PCR (Chapter 2) I have therefore chosen to use a progeny testing bioassay, rather than a PCR assay to determine the infection status of individual *M occidentalis*

To obtain the virgin individuals for the test crosses eggs or newly eclosed larvae from each of the colonies were isolated on leaf discs with prey, allowed to mature to adults, and sexed To obtain adequate numbers of females for the test crosses 50 eggs or larvae were isolated from each of the populations, with the exception of the RT populations To obtain adequate numbers of young, virgin, infected males for the test crosses, 100 individuals were isolated from each of the three RT populations, and approximately 500

more were isolated from the RT base colonies. The RT base colonies were maintained under the same conditions as the other replicate populations.

Crosses were conducted on detached pinto bean leaves on water soaked cotton. The leaves were infested with all stages of spider mites as prey. Experiments were performed under constant light at 24-27°C and 45-70% RH. Twenty, single-pair test crosses were initiated for each replicate (three replicates) of the four different populations (RT, HT, H->R, and mix), for a total of 240 crosses. Each test cross consisted of one randomly chosen RT (infected) male introduced to a randomly chosen female isolated from the various populations. They were allowed to mate, and the females were allowed to deposit eggs for a total of four days after they were determined to be gravid (signaling they have mated). The location of the newly-deposited eggs was marked daily with India ink to make relocating them easier. The number of shriveled and normal eggs, surviving progeny, and the progeny sex ratio was recorded daily. The experiment was repeated four times, at weeks 3, 6, 9, and 12, for a total of 960 test crosses. One week corresponds to one generation in *M. occidentalis*. Females which never became gravid, died, or disappeared were excluded from analysis.

Compatibility Index

Crosses were scored for the number of viable eggs, number of shriveled eggs, and sex ratio of resulting progeny. Incompatibility is associated with low numbers of viable eggs, a high degree of egg shriveling, and a strongly male-biased sex ratio (Chapter 4). A Compatibility Index was designed to include all three aspects of incompatibility when determining

infection status (the number of viable eggs ÷ number of daughters − number of shriveled eggs) ÷ 10. Crosses were scored as compatible if the Compatibility Index was greater than 0.35, and incompatible when less than 0.35. The threshold value of 0.35 clearly separated the two types based on the results of the control crosses, and corresponded to one fourth of the mean Compatibility Index value of the infected control crosses. The proportions of compatible crosses for each treatment type (RT, H->R, HT mix) were evaluated by using a simple regression analysis to check for significant deviations of the slope from zero (StatView. Abacus Concepts, 1992). An increase in compatibility over 12 generations (spread of *Wolbachia*) would be accompanied by an increase in slope.

Parameter Estimates

Unexpected incompatibility between an "infected" female and an "infected" male could mean that the *Wolbachia* was not efficiently transmitted to that female used in the cross. Likewise, unexpected compatibility between a "cured" female and "infected" male could mean that the *Wolbachia* was not efficiently transmitted to that male. To estimate the inefficiency of maternal transfer of *Wolbachia* (μ) (proportional failure of transmission), I summed the number of 'unexpected" compatible or incompatible control test crosses and divided by the total number of control test crosses.

To estimate of the hatchability "*H*" of the incompatible crosses relative to the compatible crosses, I divided the mean number of viable eggs produced by the incompatible (HT x RT and H->R x RT) crosses (H_i) by the

mean number of viable eggs produced by the compatible (RT x RT) crosses (H_c), so that $H = H_i/H_c$

Results

Progeny Testing: Control and Experimental Populations

The proportion of compatible test crosses did not increase over time in the mixed populations, indicating *Wolbachia* infection did not increase in these populations (Table 5-1). Regression analysis of the proportion of compatible test crosses from the three replicates (mixed 1, 2, and 3) over 12 generations did not indicate a slope significantly different from zero (Figure 5-1). Although the pooled data indicate no increase in compatibility, the compatibility of the mixed 1 population did increase to 24% at week 12. However, an analysis of this population alone also did not yield a slope significantly different from zero. Additionally, an increase to 24% from an initial infection frequency of 10% would not be considered the "rapid" increase in *Wolbachia* infection that has been observed in other studies.

The proportion of compatible test crosses in the RT (infected) control populations (Table 5-1) did not decrease over time, as expected. A small proportion of the test crosses were unexpectedly incompatible (1 - *proportion of compatible crosses*; Table 5-1), perhaps indicating *Wolbachia* was not perfectly transmitted to the females used in those test crosses. A similar phenomenon was noted in Chapter 4. Compatibility did not significantly increase in the crosses between the heat treated populations returned to normal rearing conditions (H->R), indicating curing by heat-treatment is stable under these

conditions. Regression analysis of the HT (cured) populations indicated the slope (0.001) was significantly different from zero, although the significance of this small increase in the proportion of compatible test crosses probably does not mean that *Wolbachia* was appearing in this heat treated line since it was kept at 33°C throughout the study.

Parameter Estimates

Table 5-1 reports the mean number of viable eggs per female per 4 days, mean number of shriveled eggs, and mean number of daughters produced for each replicate of each treatment throughout the study, as well as the proportion of the test crosses defined as compatible.

The occurrence of unexpected compatibility (e.g., HT x RT rep. 3, week 12) or incompatibility (e.g., RT x RT rep. 3, week 12) in the control crosses can be most easily explained by imperfect maternal transmission of *Wolbachia* ($\mu > 0$). There were 31 control crosses with "unexpected" compatibilities or incompatibilities out of 599 total control crosses ($\mu = 0.05$). This estimate of *Wolbachia* transmission inefficiency is within the range reported for insects ($\mu = 0 - 0.1$) (Werren, 1997).

The second parameter estimated in this experiment was hatchability (H). Using the relative numbers of viable eggs as the measure, I estimated the hatchability (H_i/H) ratio of incompatible to crosses compatible crosses as 0.11. When substituting the relative number of viable daughters produced instead of viable eggs, this hatchability estimate decreases to 0.04. This dramatic decrease in daughter production in the incompatible cross was detected in previous studies (Chapter 4; Hoy and Knop, 1981; Hoy and Standow, 1982,

Hoy and Cave 1988) and may reflect the effects of *Wolbachia* on the genetic system of *M occidentalis* (parahaploidy) In parahaploidy, both male and female embryos are diploid, but the sons lose the paternal chromosomes during embryonic development (Hoy 1979, Nelson-Rees *et al* , 1980) Since *Wolbachia*-induced incompatibilities modify the paternal set of chromosomes (Werren, 1997), the *M occidentalis* sons may be less affected by this incompatibility mechanism relative to daughters because their paternal set of chromosomes will be eliminated eventually anyway Regardless of which estimate is considered, both indicate a strong incompatibility relative to other *Wolbachia* host associations (Clancey and Hoffmann 1997)

Discussion

The bioassays conducted to estimate changes in *Wolbachia*-infection frequency over twelve generations of *M occidentalis* did not indicate a rapid increase when the initial infection frequency was 0 10 The following non-mutually exclusive reasons may explain why the *Wolbachia* infection failed to spread rapidly in these *M occidentalis* populations

Remating may be important in maintaining uninfected progeny and reducing the spread of *Wolbachia* in a population (Hoffmann *et al* , 1990) *M occidentalis* females can mate multiple times (Hoy and Smilanick 1979), which increases the chance that uninfected females will mate successfully with uninfected males within their reproductive life span.

Two assumptions inherent in the models describing the prevalence of *Wolbachia* in polymorphic populations are even (1 1) sex ratio and panmixis.

The "normal" sex ratio of *M. occidentalis* violates the assumption of a 1:1 sex ratio (Lee and Davis, 1968, Nagelkerke and Sabelis, 1991). The assumption of panmixis may also be violated which could influence the spread of the symbiont (Hoffmann and Turelli 1988). *M. occidentalis* populations are not necessarily panmictic (Hoy, 1982). Non-random mating due to behavioral mating biases also has been documented in the laboratory (Hoy and Cave 1988).

Previous studies conducted during genetic control programs of other arthropods determined that the assumptions of panmixis are often violated, and may interfere with implementation of the programs. Smith (1973) found that wild-type screwworm (*Cochliomyia hominivorax* Coquerel) females could discriminate between wild-type and sterile males. Additionally, Dieleman and Overmeer (1972) discovered that when incompatible male spider mites (*T. urticae*) were released into glasshouses in a program analogous to the sterile insect release method (SIRM), female spider mites preferred mating with compatible males rather than the incompatible males.

Perhaps the most important reason why *Wolbachia* did not increase in relative frequency in these experimental populations was that the initial infection frequency of 10% may be below an unstable equilibria frequency (="threshold frequency"). If an infection frequency is initiated below this unstable frequency *Wolbachia* may be prevented from spreading, and may actually decline to zero (Turelli and Hoffmann, 1991).

When this study was first designed, the choice of an initial infection frequency of 0.10 was influenced by a number of factors. For one, the results of Sinkins *et al.* (1995b) indicated that rapid "spread" can occur with an initial

frequency of just 10% Secondly, the feasibility of releasing more then 10% of the absolute population density in an arthropod management program is questionable Lastly the fitness costs due to infection were initially interpreted in terms of "progeny production" as has been done for the other studies using diplo-diploid insects (Stevens and Wade, 1990, Hoffmann *et al* 1990) The assumption was made that there were no relative fitness costs due to infection because the numbers of eggs produced in the infected vs the uninfected control crosses conducted in Chapter 4 were not significantly different With this in mind, Caspari and Watson (1959) predict that without fitness costs due to *Wolbachia* infection, *Wolbachia* will readily spread through a population, even at low initial frequencies

In retrospect, the number of daughters produced, rather than total progeny production, might be a more relevant measure of fecundity deficits Studies in Chapter 4 indicate that there are significant differences in the number of daughters produced in the infected control crosses relative to the uninfected control crosses Pooling the sex ratio data from control crosses in the two experiments in Chapter 4 allows an estimate of the relative fecundity (fitness) between infected (RT x RT) and uninfected (HT x HT, R->H x R->H) crosses The mean number of daughters/female in the infected control crosses was 2 94 ± 1.39 (after eliminating two incompatible crosses, see Chapter 4) The mean number of daughters/female in the uninfected control crosses was 4 86 ± 1 79 Dividing the mean daughter production of the infected control crosses by the mean daughter production of the uninfected control crosses yields a relative fitness ("F ') of 0.60 for infected crosses relative to uninfected crosses

The unstable equilibrium is very sensitive to changes in fitness costs (Hoffmann *et al*., 1990). Under the simplest model of Caspari and Watson (1959), which assumes no progeny production by incompatible crosses and perfect maternal transmission rates, the "threshold frequency" equals the fecundity cost to infected females (Werren, 1997). If we assume there are no fitness costs due to *Wolbachia* infection in *M. occidentalis* based on viable egg production alone, *Wolbachia* could spread no matter how rare it is. If the fitness costs instead are measured in terms of relative daughter production (0.6), the "threshold frequency" would be 0.40 (1 - relative fitness "F") in order for the infection to spread. This may explain why *Wolbachia* did not spread in the polymorphic populations of *M. occidentalis* in which the initial frequency of infected mites was only 0.10.

Using the parameter estimates of $\mu = 0.05$, $H = 0.11$, and $F = 0.6$ for *M. occidentalis* under laboratory conditions, the model of Hoffmann *et al*. (1990) predict an unstable equilibrium frequency of 0.50, meaning that at least 50% of the individuals needed to be infected in order for the infection to spread. Assuming no fitness costs to infection, the unstable equilibrium frequency is predicted to be 0.06, meaning that at least 6% of the individuals needed to be infected in order for the infection to spread. While the accuracy of this estimate is questionable due to violation of some assumptions, these theoretical models do provide us with hypotheses by which future experiments can be designed. Furthermore, they indicate that sex ratio may be important in determining fitness costs of *Wolbachia* infection in *M. occidentalis*, and perhaps in other parahaploid species.

The unstable equilibrium frequency of 0.50 for *M. occidentalis* is higher than the one calculated by Turelli and Hoffmann (1995) for *D. simulans* which ranged between 0.08 (with no fitness costs) and 0.19 (if relative fitness was reduced to 0.95). However other arthropod *Wolbachia* associations may also require high initial infection threshold frequencies in order for the infection to rapidly spread through a population. Stevens and Wade (1990) report that, due to fitness costs associated with the symbiont, the initial infection frequency necessary to ensure the eventual spread of their *Wolbachia* strain through laboratory populations of *Tribolium confusum* Duv. was 0.37. Clancey and Hoffmann (1997) report that, even with strong levels of incompatibility, *Drosophila serrata* transfected with a new strain of *Wolbachia* suffered enough fitness costs and had a low enough transmission efficiency that the threshold frequency would have to be approximately 0.44.

Because the required threshold frequencies can be quite high, it is proposed that stochastic events must occur in subpopulations (or "metapopulations") in order to allow the infection to exceed this unstable equilibrium frequency on a 'local' scale (Clancey and Hoffmann, 1997). These stochastic events could include drift, or founder effects (Rousset and Raymond, 1991), neighboring populations could also send out infected migrants above the threshold frequency (Turelli and Hoffmann, 1991). Phytoseid mites like *M. occidentalis* are predators of patchily-distributed spider mite prey (Sabelis and Nagelkerke, 1993), and have a subdivided metapopulation structure in which founder effects are important (Caprio and Hoy, 1994). This might allow rare infections to increase to the threshold

frequency and eventually spread through larger populations of *M. occidentalis*.

Because evolution in arthropod host *Wolbachia* associations is a dynamic process, each association should be studied individually to assess the potential for *Wolbachia* to spread through specific populations. This is especially important if a genetic control program depends on the increase of *Wolbachia*-infected individuals relative to uninfected individuals as a gene spreading mechanism. Selection is expected to occur on both hosts (Turelli, 1994) and *Wolbachia* strains (Turelli, 1994, Prout, 1994). Selection on *Wolbachia* strains would tend to favor those with the highest transmission rates and lower fecundity costs (Turelli, 1994, Prout, 1994). Such strains could, as a correlated response, then evolve to cause lower levels of incompatibilities. Strains with these attributes have been detected in *D. melanogaster* (Hoffmann *et al.*, 1994, Solignac *et al.*, 1994), and even non-sterilizing strains have been found in *D. mauritiana* Tsacas and David (Giordano *et al.*, 1995). The results of selection on hosts is more difficult to predict, but would tend to favor increased compatibility between infected males and uninfected females (Turelli, 1994).

Further investigation into the dynamics of *Wolbachia* infection in *M. occidentalis* populations would help answer some of these questions raised in this study. The biology of *M. occidentalis* violates the assumptions of equal sex ratio and panmixis. Refinement of the relevant theoretical models to make them more appropriate for the biology of *M. occidentalis* might add more sophistication to the assessment of *Wolbachia* infection dynamics in this system. However, when sex ratio was used as the measure of relative fitness,

the models appeared to support the empirical evidence obtained That the models did substantially predict the eventual outcome of this experiment supports the hypothesis that sex ratio is an appropriate measure of relative fitness in this study Studies focusing on mating biases remating potential, and metapopulation structure in laboratory colonies, and experiments conducted with higher initial infection frequencies, may provide more insight into the dynamics of *Wolbachia* infection in laboratory populations of *M occidentalis*, as well as the importance of sex ratio in this arthropod host-symbiont association

Table 5-1 Rate of *Wolbachia* spread in laboratory populations of *M accidentalis* over 12 generations evaluated by test crosses conducted at weeks 3 6, 9 and 12 Males from infected populations were crossed with 20 test females from each of the control and experimental populations The controls included three replicates each of infected (*RT*) females, heat treated (HT) (uninfected) females and heat treated females subsequently reared at room temperature (H >R) Three replicate Mixed experimental populations were initiated with 10% Infected eggs and 90% uninfected eggs

Rep	Week no	No gravid females / no producing adult daughters	Mean no./ female/ 4 d. Viable eggs (s d)	Shriveled eggs (s d)	Daughters (s d)	Proportion compatible test crosses
*Control crosses- **RT female x RT male***			*Stability of infection* [a]			
1	3	18/14	8 0 (4 5)	0 7 (1.6)	4 7 (2.9)	0 77
	6	12/12	9.5 (2.1)	0 1 (0.3)	5.8 (1 4)	0 92
	9	12/12	8 7 (1 8)	0 2 (0 4)	5.6 (1.5)	1 00
	12	18/18	10 8 (1.9)	0 1 (0.2)	7.0 (1 7)	1 00
2	3	18/17	8 9 (3 2)	0 7 (1 1)	5 1 (2 3)	0 88
	6	13/13	9 1 (2.4)	0.8 (2 4)	5 1 (2.2)	1 00
	9	12/12	9 6 (1 8)	0 (0)	5 4 (1 4)	1 00
	12	14/10	8 2 (4.9)	0.5 (0.9)	5 1 (3.5)	0.63
3	3	19/18	8 0 (3 1)	0.3 (0 1)	4 9 (2 3)	0 89
	6	17/17	10 2 (2.0)	0 (0)	6 4 (1.9)	1 00
	9	14/12	8.3 (2 9)	0.3 (0.6)	4.5 (1 9)	0 86
	12	13/11	11 8 (4.2)	0 1 (0.3)	7 7 (3 1)	0 85
*Control crosses- **H->K female x RT male***			*Stability of curing* [b]			
1	3	13/2	1 2 (1 7)	3.5 (1.8)	0.3 (0 9)	0 08
	6	20/0	0 3 (0 7)	3 6 (1.9)	0 (0)	0
	9	18/0	0 2 (0 4)	2 1 (2.2)	0 (0)	0
	12	20/4	1 6 (1 4)	3 1 (1.7)	0.2 (0 4)	0
2	3	17/4	2.7 (2.6)	4 1 (2.6)	0 6 (1.7)	0 11
	6	20/2	1 7 (2.2)	3 0 (2 2)	0.2 (0.5)	0
	9	18/2	0.8 (1 2)	1 6 (1.5)	0 2 (0 7)	0 06
	12	16/3	1.5 (1.6)	3 1 (2.5)	0 4 (1 0)	0 06
3	3	16/2	1 0 (1 3)	2 7 (1.9)	0 1 (0 3)	0
	6	17/2	0.3 (0 8)	1 2 (2.3)	0 1 (0.3)	0
	9	17/1	0 6 (0 8)	1 2 (1 8)	0 1 (0 2)	0
	12	17/1	1 6 (2 4)	2 5 (2.7)	0 4 (1 8)	0 06

Table 5-1--continued

Rep	Week no	No gravid females / no producing adult daughters	Mean no. / female / 4 d. Viable eggs (s.d.)	Shriveled eggs (s.d.)	Daughters (s.d.)	Proportion compatible test crosses
Control crosses		***HT female x RT male***	*Stability of curing* [b]			
1	3	20/4	1.1 (1.3)	4.2 (2.3)	0.3 (0.7)	0
	6	20/0	0.3 (0.7)	2.9 (1.8)	0 (0)	0
	9	18/2	1.2 (2.4)	1.6 (1.9)	0.4 (1.4)	0.06
	12	17/2	0.9 (1.4)	1.4 (1.2)	0.2 (0.5)	0.06
2	3	15/1	0.9 (1.4)	4.2 (2.5)	0.1 (0.3)	0
	6	17/1	0.9 (2.0)	1.9 (2.1)	0.3 (1.3)	0.06
	9	17/2	0.6 (0.9)	2.6 (1.9)	0.1 (0.3)	0
	12	15/3	1.7 (2.5)	2.3 (2.2)	0.7 (1.6)	0.13
3	3	17/1	0.9 (0.8)	2.7 (1.9)	0.1 (0.3)	0
	6	18/1	1.1 (1.3)	2.5 (2.1)	0.1 (0.5)	0
	9	17/4	1.0 (1.2)	1.5 (1.5)	0.2 (0.4)	0
	12	11/3	1.9 (2.7)	1.7 (1.8)	0.8 (2.1)	0.09
Experimental crosses		***Mixed female x RT male***	*Changes in infection frequency* [c]			
1	3	16/3	1.6 (2.6)	2.8 (1.8)	0.7 (1.9)	0.06
	6	18/0	0.1 (0.3)	3.3 (2.6)	0 (0)	0
	9	18/4	1.6 (2.3)	2.8 (2.6)	0.7 (1.5)	0.17
	12	17/5	2.7 (3.6)	2.5 (2.1)	1.5 (2.8)	0.24
2	3	15/6	3.6 (4.5)	1.9 (2.7)	1.9 (3.0)	0.33
	6	16/0	0.1 (0.3)	2.7 (2.4)	0 (0)	0
	9	16/1	0.9 (2.2)	0.9 (1.1)	0.4 (1.5)	0.06
	12	19/2	1.4 (2.9)	1.4 (1.5)	0.7 (2.2)	0.11
3	3	16/4	2.9 (3.7)	2.8 (2.5)	0.8 (1.6)	0.25
	6	17/4	2.1 (3.8)	1.3 (1.7)	1.4 (2.7)	0.24
	9	17/4	1.9 (2.9)	1.2 (1.9)	0.8 (1.9)	0.18
	12	16/2	1.0 (1.9)	2.1 (2.0)	0.3 (1.0)	0.06

a/ expect high proportions of compatible test crosses if infection is stable

b/ expect low proportions of compatible test crosses if curing is stable

c/ expect proportion of compatible test crosses to increase from week 3 to 12 if *Wolbachia* infection is spreading through the populations

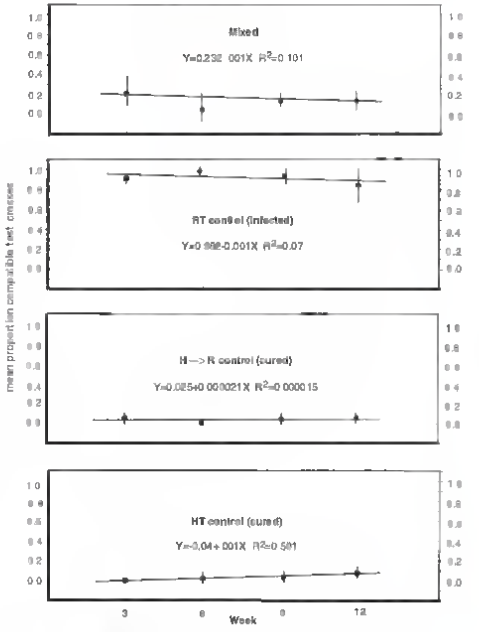


Figure 5-1 Compatibility did not increase over time in the mixed populations initiated with 10% infected and 90% uninfected individuals over 12 generations. Circles indicate means of 3 replicate populations, bars indicate standard deviation

CHAPTER 6
CONCLUSIONS

The study of *Wolbachia* symbiosis in *M. occidentalis* has been rewarding and interesting, even considering the inherent challenges of investigating an intracellular symbiont in a tiny predatory mite. The results of my research have advanced the basic understanding of this symbiosis. However, considerable work remains before the symbiosis is well understood, with numerous interesting questions remaining to be addressed.

When this research began, *Wolbachia* was suspected to be responsible for nonreciprocal reproductive incompatibilities between populations of *M. occidentalis*. This symbiont was previously described from insects, but not from mites. The use of molecular tools, including the PCR and DNA sequencing, allowed me to identify and characterize this symbiont of *M. occidentalis* in a way which was not possible before they were available.

While using these molecular tools to study the *Wolbachia* of *M. occidentalis*, I discovered that the prey of this obligate predator, the two-spotted spider mite *T. urticae*, was also infected. I then learned that research can be full of unexpected hurdles, for example, that *Wolbachia* DNA from the consumed spider mites could sometimes be detected by the PCR in uninfected predatory mites. This finding turned an already challenging molecular study into an even greater challenge. I needed to find a way to methodologically eliminate the amplification of spider mite DNA when

studying the predator's *Wolbachia*. This meant potentially risking the ability to reliably amplify the predator's intrinsic *Wolbachia* because starvation can reduce symbiont levels, and amplification from the minute eggs can be problematic. Besides the implications for my own research, the erroneous amplification of contaminating DNA from gut contents should be a consideration for anyone who studies *Wolbachia* in predatory arthropods.

It became apparent that the PCR did not always amplify the template DNA present in a sample, even though "theoretically" it could amplify even a single molecule. Semi-nested PCR and/or pooling of DNA samples was often necessary to get a PCR signal, and still these techniques occasionally failed. This difficulty of detecting minute amounts of *Wolbachia* in single tiny predators was another obstacle that needed to be addressed throughout this study.

In retrospect, more techniques should have been attempted to improve the sensitivity of the PCR. Lack of experience led me to assume that PCR amplification was straightforward for other research groups studying *Wolbachia*, and that the techniques used in my own lab to study mite DNA were adequate for symbiont DNA from the mites. I have realized this was not the case. When problems arose, these assumptions led me to question my technical abilities, rather than the techniques themselves. If I had accepted earlier that the study of *Wolbachia* in mites was inherently difficult, I might have attempted more techniques. For example, I might have tried different extraction techniques such as a CTAB protocol, or concentrated more efforts on improving the STE procedure (O'Neill *et al.* 1992, Chapter 2) which initially appeared to work more reliably than the Chelex method (Chapter 2).

Increasing the number of PCR cycles also might have been helpful, as has been recently recommended for reactions with low template concentrations (Rameckers *et al* , 1997) This experience taught me that when techniques do not work well, even "established" ones alternative approaches should be explored

In spite of these problems, interesting data resulted from this research For one, the DNA sequences I examined from the 16S ribosomal RNA and *ftsZ* genes from the predator and prey were nearly identical to each other Unexpectedly, the sequences from the mite *Wolbachia* were nearly identical to the *Wolbachia* from insects, including the type species *Wolbachia pipientis* from the mosquito *Culex pipiens* Whether the *Wolbachia* from the mites are truly this similar to each other and to the symbionts from insects remains to be answered The genes I used were too conserved to resolve this question

Information on sequence variation would have been very useful in designing species-specific primers, so I could more easily eliminate false positives from the predator's gut contents The possibility that this maternally transmitted symbiont has been transferred horizontally between the two mite species also remains unanswered because of the lack of sequence variation Perhaps a more variable portion of the *ftsZ* gene should have been studied, like the non coding region, or even an entirely different gene Information was available on the non-coding region of the *ftsZ* gene from an A-group *Wolbachia* from *Drosophila melanogaster* (Holden *et al* , 1993) Designing primers for that region which would be *Wolbachia*-specific and would work on the B-group *Wolbachia* from *M occidentalis* and *T urticae* would have been time consuming, but might have yielded useful information

on the genetic variation between *Wolbachia* sequences. Genetic information on this microorganism has been scarce, but continual advances are expected due to the exponential growth of *Wolbachia* research around the world and a new *in vitro* culturing system in a mosquito cell line (Werren, 1997).

In order to study the biological effects of *Wolbachia* infection, it is crucial to obtain a population without the symbionts with which infected individuals can be crossed or compared. In other studies, the insects cooperate by consuming antibiotic-laced artificial diet, honey, or water. In view of the difficulty in feeding antibiotics to these obligate predators, the ability to "cure" these mites by rearing them at high temperatures was an exciting option. This allowed me to determine the phenotype of *Wolbachia*-induced reproductive incompatibilities between infected males and uninfected females. Interestingly, the phenotype was a unique combination of reduced progeny production (as in diplo-diploids) and a skewed sex ratio (as in haplo-diploids) of the few, resulting progeny. However, one nagging question remains. Are correlations between the presence of *Wolbachia* and nonreciprocal incompatibilities just that? Is another, heat-sensitive cytoplasmic element the real cause of the incompatibilities? The evidence from this study, and studies with so many other arthropods, strongly suggests it is the *Wolbachia*. However, until a *pure* sample of *Wolbachia* can be *successfully* injected into uninfected mites, this possibility cannot be excluded. According to Koch's postulates, reinfection of the suspected microorganism is a necessary step in the assignment of cause and effect.

The crossing studies yielded another interesting and unexpected result. The infected control crosses tended to have a male-biased sex ratio, and the

uninfected crosses had a female-biased sex ratio If this is a general phenomenon and not just a sampling error it could have interesting and important implications for the population dynamics of this agriculturally-important mite by altering its intrinsic rate of increase

The "infected" control crosses occasionally produced some unexpected incompatible crosses The efficiency of symbiont transmission from mother to progeny appears to be imperfect, due to unknown factors which could include crowding and nutritional stresses Imperfect transmission or other reductions of symbiont titer might contribute to unexpected within-population incompatibilities if some females do not have enough *Wolbachia* to be compatible with an infected male This underscores the importance of reducing colony crowding and other stresses when rearing large numbers of infected *M occidentalis* or other natural enemies in biological control programs Because *Wolbachia* or other symbioses have the potential to affect entomological projects in positive and negative ways I believe that they should be an important consideration when addressing the biology of any arthropod Symbioses transform an individual into a community complete with a fascinating complexity of interactions we are only beginning to understand

The results of the *Wolbachia* infection dynamics studies were some of the most surprising and important findings In contrast to published theoretical predictions and empirical evidence that *Wolbachia* will spread rapidly through polymorphic populations this did not occur in populations of *M. occidentalis* There may be several non-mutually exclusive reasons why this symbiont which should have spread did not Perhaps it was because the

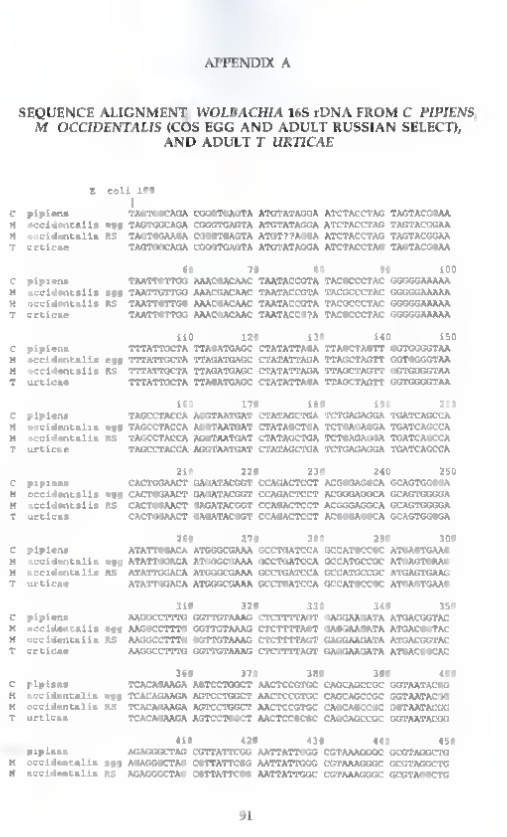
initial infection frequency of 10% was below a threshold which would allow the frequency to increase to a stable level. It would have been interesting to create some populations with a higher initial infection frequency than 10% and compare the outcomes. Another possible reason why *Wolbachia* did not rapidly spread through the populations could have been due to fitness costs associated with infection, like reduced female sex ratio compared to cured mites. Perhaps the females will mate multiple times if they detect they are producing shriveled eggs. This would increase the chances of uninfected females producing viable uninfected offspring. This could then reduce the "spread" of infected individuals by maintaining the presence of more uninfected individuals in the population. Addressing these questions would not only contribute to our basic knowledge of *M. accidentalis* biology, but might have implications for those hoping to use *Wolbachia* as a "drive mechanism".

There are still many mysteries concerning the symbiotic relationship between *Wolbachia* and arthropod hosts. For example, recent investigations indicate *Wolbachia* infection is responsible for protecting a weevil from a parasitoid (Hsiao, 1996). This phenomenon could have widespread implications for the way scientists match pest biotypes with natural enemy biotypes in biological control programs. Ecological implications of this symbiosis are important in another biological control setting. There are plans to infect parasitoid wasps with *Wolbachia* so that they only produce daughters, since the ovipositing females are the "effective" natural enemies. While the relative number of ovipositing females could be increased this way,

there could be serious fitness effects due to infection, or detrimental consequences from the loss of genetic recombination (Crow 1988)

I enjoyed answering some of the questions regarding *Wolbachia* symbiosis in *M occidentalis*, even considering the challenges inherent in this project There are many interesting questions which remain The following are three which would be most interesting to answer First, it would be interesting to use a more variable *Wolbachia* gene to determine the differences between the symbionts from the predator and prey and between mites and insects. Second, a detailed life table analysis would be important to more accurately determine any fitness costs of *Wolbachia* infection Finally, investigating the females' responses to laying shriveled eggs after mating with incompatible, infected males would be important in understanding the dynamics of *Wolbachia* infection in polymorphic populations The answers to these questions, in addition to those already answered in this research, will provide a better understanding of *Wolbachia* symbiosis in the agriculturelly-important predatory mite *Metaseiulus occidentalis* and possibly other *Wolbachia* symbioses as well The inevitable cascade of questions, answers, and more questions is one of the most important lessons learned during this project It is one of the most frustrating, and well as one of the most exciting aspects of science

SEQUENCE ALIGNMENT, *WOLBACHIA* 16S rDNA FROM *C. PIPIENS*, *M. OCCIDENTALIS* (COS EGG AND ADULT RUSSIAN SELECT), AND ADULT *T. URTICAE*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M occidentalis RS TAGTGGAAGA CGGGTGAGTA ATGTTTAGGA ATCTACCTAG TAGTACGGAA
T urticae TAGTGGCAGA CGGGTGAATA ATGTATAGGA ATCTACCTAG TAGTACGGAA
60# 70# 80# 90# 100
C pipiens TAATTGTTGG AAACGACAAC TAATACCGTA TACGCCCTAC GGGGGAAAAA
M occidentalis wgg TAATTGTTGG AAACGACAAC TAATACCGTA TACGCCCTAC GGGGGAAAAA
M occidentalis RS TAATTGTTGG AAACGACAAC TAATACCGTA TACGCCCTAC GGGGGAAAAA
T urticae TAATTGTTGG AAACGACAAC TAATACCGTA TACGCCCTAC GGGGGAAAAA
110 120 130 140 150
C pipiens TTTATTGCTA TTAGATGAGC CTATATTAGA TTAGCTAGTT GGTGGGGTAA
M occidentalis wgg TTTATTGCTA TTAGATGAGC CTACATTAGA TTAGCTAGTT GGTGGGGTAA
M occidentalis RS TTTATTGCTA TTAGATGAGC CTATATTAGA TTAGCTAGTT GGTGGGGTAA
T urticae TTTATTGCTA TTAGATGAGC CTATATTAGA TTAGCTAGTT GGTGGGGTAA
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C pipiens TAGCCTACCA AGGTAATGAT CTAGAGCTGA TCTGAGAGGA TGATCAGCCA
M occidentalis wgg TAGCCTACCA AGGTAATGAT CTATAGCTGA TCTGAGAGGA TGATCAGCCA
M occidentalis RS TAGCCTACCA AGGTAATGAT CTATAGCTGA TCTGAGAGGA TGATCAGCCA
T urticae TAGCCTACCA AGGTAATGAT CTAGAGCTGA TCTGAGAGGA TGATCAGCCA
210 220 230 240 250
C pipiens CACTGGAACT GAGACACGGT CCAGACTCCT ACGGGAGGCA GCAGTGGGAA
M occidentalis wgg CACTGGAACT GAGATACGGT CCAGACTCCT ACGGGAGGCA GCAGTGGGGA
M occidentalis RS CACTGGAACT GAGATACGGT CCAGACTCCT ACGGGAGGCA GCAGTGGGGA
T urticae CACTGGAACT GAGATACGGT CCAGACTCCT ACGGGAGGCA GCAGTGGGGA
260 270 280 290 300
C pipiens ATATTGGACA ATGGGCGAAA GCCTGATCCA GCCATGCCGC ATGAGTGAAG
M occidentalis wgg ATATTGGACA ATGGGCGAAA GCCTGATCCA GCCATGCCGC ATGAGTGAAG
M occidentalis RS ATATTGCACA ATGGGCGAAA GCCTGATCCA GCCATGCCGC ATGAGTGAAG
T urticae ATATTGGACA ATGGGCGAAA GCCTGATCCA GCCATGCCGC ATGAGTGAAG
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M occidentalis RS AAGGCCTTTG GGTTGTAAAG CTCTTTTAGT GAGGAAGATA ATGACGGTAC
T urticae AAGGCCTTTG GGTTGTAAAG CTCTTTTAGT GAGGAAGATA ATGACGCCAC
360# 370# 380# 390# 400#
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M occidentalis RS TCACAGAAGA AGTCCTGGCT AACTCCGTGC CAGCAGCCGC GGTAATACGG
T urticae TCACAGAAGA AGTCCTGGCT AACTCCGCGC CAGCAGCCGC GGTAATACGG
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C pipiens AGAGGGCTAG CGTTATTCGG AATTATTGGG CGTAAAGGGC GCGTAGGCTG
M occidentalis wgg AGAGGGCTAG CGTTATTCGG AATTATTGGG CGTAAAGGGC GCGTAGGCTG
M occidentalis RS AGAGGGCTAG CGTTATTCGG AATTATTGGC CGTAAAGGGC GCGTAGGCTG

```
T urticae              [illegible] ATTCGG AATTATTCAC [illegible]

                                 460        470        480        490        500
C pipiens              GTTAATAAGT TAAAAGTGAA ATCCCGAGGC TTAACCTTGG AATTGCTTTT
M occidentalis egg     GTTAATAAGT TAAAAGTGAA ATCCCGAGGC TTAACCTTGG AATTGCTTTT
M occidentalis RS      GTTAATAAGT TAAAAGTGAA ATCCCGAGGC TTAACCTTGG AATTGCTTTT
T urticae              ?????????? TAAAAGTGAA ATCCCGAGGC TTAACCTTGG AATTGCTTTT

                                 510        520        530        540        550
C pipiens              AAAACTATTA ATCTAGAGAT TGAAAGAGGA TAGAGGAATT CCTGATGTAG
M occidentalis egg     AAAACTATTA ATCTAGAGAT TGAAAGAGGA TAGAGGAATT CCTGATGTAG
M occidentalis RS      AAAACTATTA ATCTAGAGAT TGAAAGAGGA TAGAGGAATT CCTGATGTAG
T urticae              AAAACTATTA ATCTAGAGAT TGAAAGAGGA TAGAGGAATT CCTGATGTAG

                                 560        570        580        590        600
C pipiens              AGGTAAAATT CGTAAATATT AGGAGGAACA CCAGTGGCGA AGGCGTCTAT
M occidentalis egg     AGGTAAAATT CGTAAATATT AGGAGGAACA CCAGTGGCGA AGGCGTCTAT
M occidentalis RS      AGGTAAAATT CGTAAATATT AGGAGGAACA CCAGTGGCGA AGGCGTCTAT
T urticae              AGGTAAAATT CGTAAATATT AGGAGGAACA CCAGTGGCGA AGGCGTCTAT

                                 610        620        630        640        650
C pipiens              CTGGTTCAAA TCTGACGCTG AAGCGCGAAG GCGTGGGGAG CAAACAGGAT
M occidentalis egg     CTGGTTCAAA TCTGACGCTG AAGCGCGAAG GCGTGGGGAG CAAACAGGAT
M occidentalis RS      CTGGTTCAAA TCTGACGCTG AAGCGCGAAG GCGTGGGGAG CAAACAGGAT
T urticae              CTGGTTCAAA TCTGACGCTG AAGCGCGAAG GCGTGGGGAG CAAACAGGAT

                                 660        670        680        690        700
C pipiens              TAGATACCCT GGTAGTCCAC GCTGTAAACG ATGAATGTTA AATATGGGGA
M occidentalis egg     TAGATACCCT GGTAGTCCAC GCTGTAAACG ATGAATGTTA AATATGGGGA
M occidentalis RS      TAGATACCCT GGTAGTCCAC GCTGTAAACG ATGAATGTTA AATATGGGGA
T urticae              TAGATACCCT GGTAGTCCAC GCTGTAAACG ATGAATGTTA AATATGGGGA

                                 710        720        730        740        750
C pipiens              GTTTACTTTC TGTATTACAG CTAACGCGTT AAACATTCCG CCTGGGGACT
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M occidentalis RS      GTTTACTTTC TGTATTACAG CTAACGCGTT AAACATTCCG CCTGGGGACT
T urticae              GTTTACTTTC TGTATTACAG CTAACGCGTT AAACATTCCG CCTGGGGACT

                                 760        770        780        790        800
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M occidentalis RS      ACGGTCGCAA GATTAAAACT CAAAGGAATT GACGGGGACC CGCACAAGCG
T urticae              ACGGTCGCAA GATTAAAACT CAAAGGAATT GACGGGGACC CGCACAAGCG

                                 810        820        830        840        850
C pipiens              GTGGAGCATG TGGTTTAATT CGATGCAACG CGAAAAACCT TACCACTTCT
M occidentalis egg     GTGGAGCATG TGGTTTAATT CGATGCAACG CGAAAAACCT TACCACTTC
M occidentalis RS      GTGGAGCATG TGGTTTAATT CGATGCAACG CGAAAAACCT TACCACTTCT
T urticae              GTGGAGCATG TGGTTTAATT CGATGCAACG CGAAAAACCT TACCACTTCT

                                 860
C pipiens              TG
M occidentalis egg
M occidentalis RS
T urticae              TG
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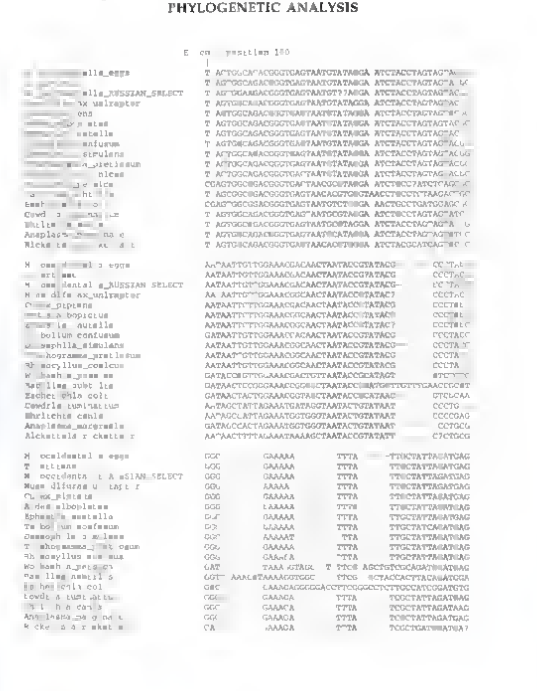
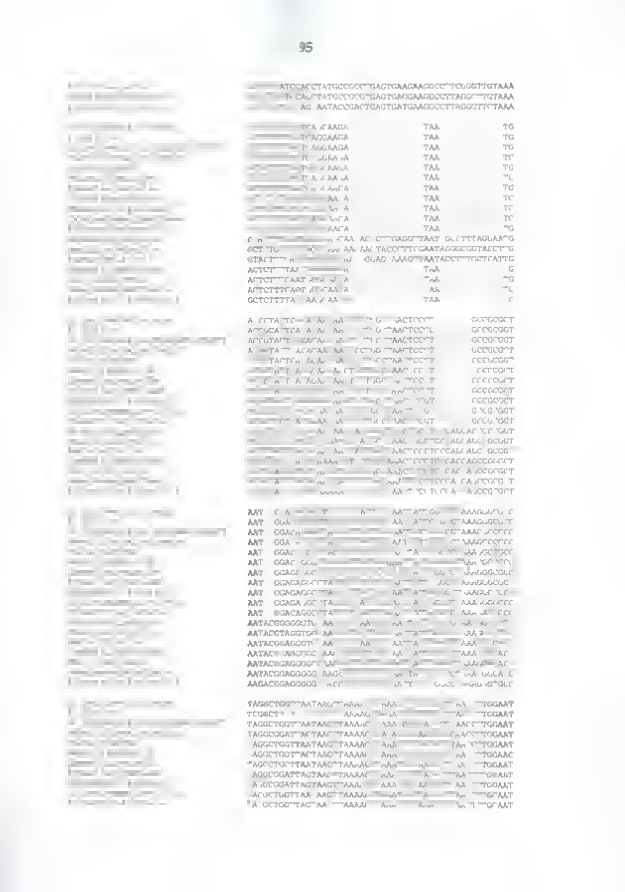
APPENDIX B

SEQUENCE ALIGNMENT *WOLBACHIA* 16S rDNA FOR PHYLOGENETIC ANALYSIS





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